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EXPERIMENTAL, MILD BLAST-INDUCED TRAUMATIC BRAIN INJURY: FOCUS ON THE MONOAMINE AND GALANIN SYSTEMS

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Cover image: *In situ* hybridisation photomicrograph of Galanin Receptor 1 in the rat ventral periaqueductal grey, digitally manipulated.

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Experimental, mild blast-induced traumatic brain injury: focus on the monoamine and galanin systems

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Dedicated to my mother.

Mum, I aspire to have your courage and perseverance.

With kind permission from the Kurdish painter, Omar Delware.

“All war is a symptom of man's failure as a thinking animal.”
— John Steinbeck

ABSTRACT

Traumatic brain injury (TBI) is a common cause of mortality and morbidity among civilians and servicemen alike. The spectrum of TBIs encompasses mild to severe cases, with the predominate number of TBIs being mild (mTBI). Combat-related TBI inflicted by explosive blast (bTBI) is highly prevalent among military personnel. The injurious environment caused by explosive blast includes the high-energy shock wave that dissipates energy at the boundaries of anatomical structures with distinct acoustical impedance, and the blast wind that can propel the body resulting in acceleration/ deceleration type of injury. mTBI can also occur in the civilian population often as a result of contact sports and because of the mild acute symptoms it is often repeated. mTBI causes transient mood and cognitive changes, but it may also increase the risk for late onset chronic neurodegenerative conditions such as chronic traumatic encephalopathy. Little is known about the mechanism of mTBI and its post-sequelae. Monoamine projections from the brainstem play a key role in modulating the forebrain regions, and galanin, a neuropeptide that, in the rat, is co-localised with two of these key neurotransmitters. Dysfunctions in these systems have been associated with mood/anxiety disorders.

In this thesis we set out to examine changes in the monoamine and galanin systems following single and repeated blast exposure, in male and female rats. mTBI was induced using an experimental blast tube which uses real explosives. For one of the studies a shock tube that uses compressed air was also used to produce a blast-induced mTBI (mbTBI). The models appeared to cause mbTBI, given that no injury was observed when staining for degeneration, blood vessel damage, or disruption to the white matter tracts in either model, following single or repeated exposure.

The noradrenaline (NA) system was found to be particularly sensitive to mbTBI. The transcript levels of the biosynthetic enzyme tyrosine hydroxylase (TH) were found elevated immediately post-exposure bilaterally in the locus coeruleus (LC) in both males and females. This was concurrent with a transient increase in NA levels in a number of forebrain regions, and translated into decreased immobility in the forced swim test. This was only explored in the males and using the blast tube.

Sex-specific differences were found in the serotonin system (5-HT). Here the transcript levels of the biosynthetic enzyme tryptophan hydroxylase 2 (TPH2), were elevated across the mid/caudal-rostral dorsal raphe nucleus (DRN) in females. The elevation occurred acutely post-TBI and remained even after day (D)7, the last time point evaluated. In the males TPH2 was similarly elevated, but more modestly and only transiently. The increase was limited to only the mid/caudal part of the DRN, and by D3 TPH2 levels were similar to levels detected in sham animals. No changes in 5-HT levels were seen in the forebrain regions of male rats.

Exposure induced changes in the expression of galanin and its receptors were also examined by *in situ* hybridisation but in males only. Galanin mRNA levels increased bilaterally in the LC and gradually in the mid/caudal, but not in the rostral DRN and remained elevated, even at D7 in both nuclei.. However, quantitative polymerase chain reaction only confirmed the acute galanin increase in the LC, and in addition revealed galanin transcripts in the hippocampus. In terms of the galanin receptor 1-3 (GalR1-3), GalR1 was increased in the ventral periaqueductal grey and this increase persisted at D7 post-TBI, at this time-point GalR2 was decreased. In the forebrain regions GalR3 appeared to be the most dynamic receptor, decreasing in most regions immediately following TBI, and recovering on D7 post-exposure, except in the ventral hippocampus, where changes persisted.

A cumulative effect of repeated exposure to blast was not apparent in the levels of the transcripts for TH, TPH2, and galanin using either the blast or shock tube in male rodents.

Serum analyses revealed sex-specific differences acutely following a single blast exposure, including an increase in corticosterone and substance P in female and decreased BDNF levels in male exposed rats.

Taken together, these findings indicate a role for both the monoamine and the galanin systems following blast exposure, these changes appear robust across models and sexes (although some sex-specific differences are apparent). Hence these systems are possibly mediators of post-TBI sequelae and targeting the persistent dysfunctions in these systems may bring about therapeutic benefits.

LIST OF SCIENTIFIC PAPERS

Paper I. Kawa, L., Arborelius, U., Yoshitake, T., Kehr, J., Hökfelt, T., Risling, M., Agoston, D., (2014). Neurotransmitter systems in a mild blast traumatic brain injury model: catecholamines and serotonin. J. Neurotrauma 32, 1190–9.

Paper II. Kawa, L., Barde, S., Arborelius, U.P., Theodorsson, E., Agoston, D., Risling, M., Hökfelt, T., (2016). Expression of galanin and its receptors are perturbed in a rodent model of mild, blast-induced traumatic brain injury. Exp. Neurol. 279, 59–67

Paper III. Kawa, L., Kamnaksh, A., Long, J.B., Arborelius, U.P., Hökfelt, T., Agoston, D., Risling, M., (2016). A comparative study of two blast-induced traumatic brain injury models: Changes in monoamine and galanin systems following single and repeated exposure (manuscript)**.**

Paper IV. Kawa, L., Angeria, M., Arborelius, U.P., Hökfelt, T., Risling, M (2016). Exploring sex-specific differences in rodents following a mild blast-induced traumatic brain injury (manuscript)**.**

CONTENTS

LIST OF ABBREVIATIONS

5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
AADC	Aromatic acid decarboxylase
Amg	Amygdala
ACh	Acetylcholine
AOC	Alterations of consciousness
APP	β-amyloid precursor protein
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
bTBI	Blast-induced traumatic brain injury
CCK	Cholecystokinin
CNS	Central nervous system
CORT	Corticosterone
CT	Computed tomography
CX	Occipital cortex
DA	Dopamine
dHiFo	Dorsal hippocampal formation
DTI	Diffusion tensor imaging
DOPAC	3,4-dihydroxyphenylacetic acid
DRN	Dorsal raphe nucleus
ERC	Entorhinal cortex
FJ	Fluoro jade B
FST	Forced swim test
GalR1-3	Galanin receptor 1-3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCS	Glasgow coma scale
HPA	Hypothalamic–pituitary–adrenal axis
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
Hyp	Hypothalamus
IHC	Immunohistochemistry
IMMO	Immobilisation
ISH	In situ hybridisation
LC	Locus coeruleus
LOC	Loss of consciousness
MGD	Mean grey density
MRI	Magnetic resonance imaging
MRN	Median raphe nucleus
mRNA	Messenger ribonucleic acid
mTBI	Mild traumatic brain injury
MWM	Morris water maze
NA	Noradrenaline
NPY	Neuropeptide Y
OF	Open field
P-/TAU	Phospho/native tubulin-associated protein
PFC	Prefrontal cortex
PNS	Peripheral nervous system
PTA	Post-traumatic amnesia
PTSD	Post-traumatic stress disorder

qPCR	Quantitative polymerase chain reaction
RIA	Radioimmunoassay
ROI	Region of interest
S100B	S100 calcium-binding protein B
SP	Substance P
TBI	Traumatic brain injury
TH	Tyrosine hydroxylase
TPH2	Tryptophan hydroxylase 2
VEGF	Vascular endothelial growth factor
vHiFo	Ventral hippocampal formation
vPAG	Ventral periaqueductal grey

1 INTRODUCTION

1.1 Traumatic Brain Injury

1.1.1 Blast-related TBI

Traumatic brain injury (TBI) sustained during military-related combat in recent conflicts has largely been a result of explosive devices including: artillery, mines, booby traps, aerial bombs, improvised explosive devices, and rocket propelled grenades (Warden, 2006). In previous wars exposure to such weaponry would have likely resulted in death. Improvements in body armour and on the front-line medical care have increased survival rates (Okie, 2005; Purim-Shem-Tov et al., 2013). While the protective gear effectively shields a soldier from shrapnel and bullets, it cannot completely protect against the face, neck, and closed head injuries, typical of a blast exposure (Xydakis et al., 2005). In fact, studies estimate that approximately 56-78% of head injuries inflicted in the current wars were a result of blast exposure (Murray et al., 2005; Sayer et al., 2008). TBI in this context is termed blast-induced TBI (bTBI), which is a complex incident and can be divided into a number of events (Figure 1.1).

The instantaneous conversion of an explosive into gas during a detonation results in rapid changes in atmospheric pressure. Initially, there is an immediate increase in pressure due to heating and accelerating of air molecules, followed by a sudden drop (Katherine H. Taber et al., 2014; Moore and Jaffee, 2010). These extreme pressure changes can result in the *primary injury* when the blast wave reaches the body, an initial burst of energy can be transmitted through the head and into the brain (Risling and Davidsson, 2012). *Secondary injury* can be caused by the force of the blast winds, which can propel shrapnel and other fragments into motion with considerable force. Impact to the head can result in either blunt force or penetrating injury to the brain. *Tertiary injuries* occur as a result of the kinetic energy generated and released by the blast wind, which accelerates the body. Once the body comes to a stop, the brain continues to move in the direction of the force and thus collides with the tough skull bone, shearing and diffuse injuries likely results. Finally, *quaternary injuries* can include flash burns and breathing in noxious gases associated with the explosion (Cernak, 2010; Risling and Davidsson, 2012). Additionally, blast exposure is systemic and impacts the whole body, thus air emboli can form in the blood vessels elsewhere and be transported up to the brain (Cernak, 2010; Guy et al., 2000).

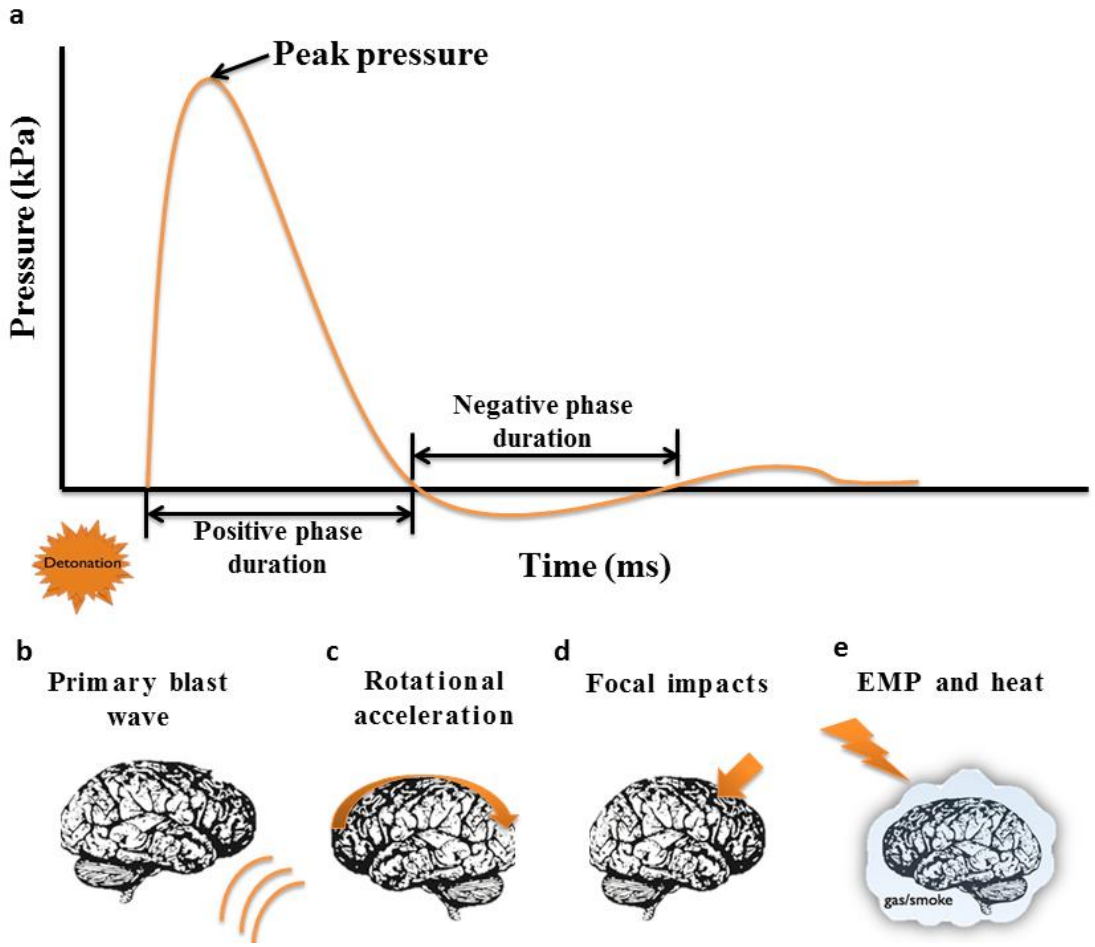

Figure 1.1 Blast-related TBI. a) Sequence of changes in atmospheric pressure following a detonation. (b-d) Schematic representation of the different injury mechanisms following blast exposure.

1.1.2 Use of animal models

The aforementioned series of injuries seldom occur in isolation, and in a battlefield situation it can be extremely difficult to ascertain the different injury mechanisms. Moreover, clinically we have limited knowledge about how the physics of a blast exposure interacts with a biological system. Here the use of animal models can be quite beneficial. The models can be used to recapitulate the different injury mechanism following a blast exposure in a reproducible manner, allowing researchers to study and dissect each mechanism of injury independently. Animal models therefore enable researchers to tackle the complexity and multifaceted nature of a blast exposure in amenable steps.

There are some obvious caveats to using animal models, particularly rodents. There are questions about scalability given rodents' brains compared with our gyrencephalic ones, which likely differ in interacting with a blast wave. Furthermore, the biological clock in rodents is significantly different to humans. These limitations need to be considered when

attempting to translate findings across rodent models to humans. However, several factors have made rodents the model of choice, including cost-effectiveness, ability to manipulate and intervene with the disease progression, and availability of numerous behavioural assays, as well as, especially for the mouse, to generate genetically manipulated animals.

Two of the most commonly used animal models to reproduce a segment of the blast-induced TBI, the *primary blast wave* exposure, are the shock tube and blast tube. The shock tube is comprised of a compression and expansion chamber, separated by mylar membranes of varying thickness. Once air or helium are pressurised in the compression chamber above the threshold of the designated mylar membrane(s), it ruptures and results in a pressure wave inside the expansion chamber (Long et al., 2009a; Ning and Zhou, 2015). The blast tube uses real explosives as the driver, and upon detonation a blast wave is propagated (Clemedson and Criborn, 1955).

The interaction between a rapidly propagating, high-energy blast wave and the brain's varying structure and density is likely to mean exposure to the primary blast wave alone and can trigger an array of complex cellular and molecular changes. Indeed, findings from both models have revealed the brain's vulnerability to the primary blast wave (Ahlers et al., 2012; Elder et al., 2014; Kamnaksh et al., 2012; Kwon et al., 2011; Risling et al., 2011; Wang et al., 2011). This thesis will focus on the primary blast exposure predominantly using the blast tube using rats, but the shock tube is also used.

1.1.3 Classification of TBI

TBI is a spectrum disorder, ranging between mild, moderate, and severe. In both the military and civilian population, the significant majority of TBIs (typically around 85%) are mild in severity (mTBI) (Hyder et al., 2007). In the civilian population the majority of mTBI is a result of contact sports and is frequently referred to as concussion. mTBI and concussion are often used interchangeably. The most common tests used to identify the severity of a brain injury are:

I. the Glasgow Coma Scale (GCS), which assesses best eye, verbal and motor responses
II. any decreased or Loss of Consciousness (LOC) caused by the injury
III. any Alterations in Consciousness (AOC)
IV. alterations in memory immediately preceding or after the event, termed Post-traumatic Amnesia (PTA).

A mild TBI is characterised by a GCS score of 13 to 15 (Figure 1.2), LOC of up to but not exceeding 30 minutes, and AOC and PTA for less than 24 hours (CDC, 2003; Teasdale and Jennett, 1974; VA/DoD, 2009). But these tools do not provide objective evidence of brain injury and are thus poor correlates of injury progression or recovery. In addition, common neuroimaging scans such as computed tomography (CT) are almost always normal i.e. currently there are no detectable structural changes in the brain post-trauma with

the diagnostic tools readily available to physicians. Hence, making mTBI diagnosis particularly challenging and reliant on patient re-call, which is often biased and incomplete (Drake et al., 2010).

In the military setting, new tools have been developed in the form of neuropsychological testing to aid in the diagnosis of mTBI in soldiers (Reeves et al., 2007). As of 2008 the United States Armed Forces requires service members to complete neuropsychological tests pre-deployment to ascertain their baseline neurocognitive evaluations using an Automated Neuropsychological Assessment Metric (ANAM) (VA/DoD, 2009). ANAM can be re-administered following injury. If scores are compared to the baseline, impairments can be identified (and hence concussed from non-concussed individuals). Following a possible injury, the military acute concussion evaluation (MACE), (another screening tool) can also be used immediately in theatre, administered by a skilled practitioner. Initial evaluations of these screening tools for detecting concussion in soldiers looks promising, particularly the use of ANAM (Kelly et al., 2012).

Glasgow Coma Scale		Score
Best eye response	Spontaneous	4
	Opens to verbal command	3
	Opens to pain	2
	No response	1
Best verbal response	Orientated to time, place and person	5
	Confused/disorientated	4
	Inappropriate responses	3
	Incomprehensible speech	2
	No response	1
Best motor response	Obeys commands	6
	Moves to localised pain	5
	Withdraws from pain	4
	Abnormal flexion	3
	Abnormal extension	2
	No response	1

Figure 1.2 The Glasgow Coma Scale comprising of three tests: eye, verbal, and motor responses. The sum of the lowest possible GCS is 3 (deep coma or death), while the highest is 15 (fully awake person).

1.1.4 Post-concussive symptoms

Following a mTBI/concussion a wide spectrum of neurobehavioural symptoms can develop, which can have acute onset and be transient, or develop over time and become more chronic (Marion et al., 2011). While for the majority of those who suffer a mTBI there are no symptoms past 24 hours, some 10-15% have lingering symptoms which persist months or years post-injury (Tanielian et al., 2008; VA/DoD, 2009). These symptoms largely fall into three categories: emotional, cognitive, and somatic disturbances, and are collectively referred to as post-concussive syndrome (PCS). A detailed list of categorised symptoms is shown in Figure 1.3. PCS can adversely affect the performance and overall quality of life of an affected individual and is a source of morbidity.

Descriptions of lingering symptoms following blast exposure that left soldiers dazed and confused, with no physical evidence of external trauma to the head, were described and met with considerable interest in the early twentieth century, following World War I (WWI) (Mott, 1919; Myers, 1915). *Shell-shock* was one of the terms then coined referring to a syndrome that encompassed much of the symptomology that is part of PCS (Mott, 1919). This term was abandoned following an inauspicious report commissioned by the British Government, which declared shell-shock as a "convenient evasion of duty if not disguised malingering."

Concerns have since been renewed following several reports of persistent traumatic sequelae following a mTBI in both the military and sports populations (Colvin et al., 2009; Gardner and Yaffe, 2015; Kontos et al., 2013; Mendez et al., 2013; Rosenfeld and Ford, 2010). Some of the clinical aspects of PCS can be recapitulated in experimental models of blast-induced TBI using rodents, such as those relating to cognition (e.g. learning and memory) and emotional impairments (e.g. anxiety and depression) (Budde et al., 2013; Genovese et al., 2013; Heldt et al., 2014; Kamnaksh et al., 2012; Kwon et al., 2011). This thesis will focus on the emotional impairments following a blast exposure.

Post-concussive symptoms

Emotional

- Depression
- Anxiety
- Irritability
- Mood changes

Cognitive

- Memory problems
- Concentration problems
- Decision making problems

Somatic

- Headache
- Fatigue
- Visual/hearing disturbances
- Dizziness
- Sleeping disturbances
- Nausea

Figure 1.3 Emotional, cognitive, and somatic impairments reported following mTBI.

1.1.5 Post-traumatic stress disorder

Post-traumatic stress disorder (PTSD) is a complex disease triggered by exposure to extreme psychological stress, frequently without physical injury. Symptoms are commonly organised into four clusters and include avoidance symptoms, hyperarousal and reactivity, re-experiencing phenomena, and mood and cognition impairments (Heim and Nemeroff, 2009; Pitman et al., 2012). PTSD involves substantial symptom overlap with PCS, and is commonly contaminant with mTBI in the military population (Hoge et al., 2004; Kennedy et al., 2010; Kok et al., 2012).

Clinical studies suggest that PTSD prevalence is far higher in the military compared to the civilian population, and sustaining a TBI is a risk factor (Hoge et al., 2008; Kennedy et al., 2010; Kessler et al., 1995; Schneiderman et al., 2008). Interestingly, a study looking at the severity of injury and risk, found that PTSD is more prevalent in patients with a mTBI in comparison to individuals with more severe TBIs (Kennedy et al., 2010). The authors suggested that the lack of detectable physical injury and the vague and ambiguous trajectory of recovery in mTBI lead to increased onset of anxiety and other PTSD-related symptoms. Animal models have recapitulated some PTSD-related behavioural traits following repeated blast exposure. Exposed rats exhibited increased startle response, and increased anxiety six weeks post-exposure, concurrent with elevated stathmin 1 protein in the amygdala (Elder et al., 2012). Another study also induced increased acoustic startle, and enhanced contextual fear response 2-8 weeks following blast-exposure in mice (Heldt et al., 2014).

1.1.6 Neurochemical cascade following TBI

Following TBI there is an abrupt interference of cellular homeostasis, triggering a number of biochemical alterations in the brain. Some of these changes are summarised in Figure 1.4. Significant mechanical stress can cause shearing and stretch forces that in turn cause disruption to cellular membranes, known as mechanoporation, resulting in an efflux of K^+ (Farkas et al., 2006). The efflux of K^+ causes neuronal depolarisation and firing, and indiscriminate release of neurotransmitters, mainly excitatory amino acids (EEAs) (Katayama et al., 1990). Thus there is a large increase in extracellular concentrations of glutamate (Katayama et al., 1990). EEAs can bind to a number of receptors and channels including n-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid *(*AMPA) receptors (Faden et al., 1989). The engagement of these receptors and opening of more voltage gated channels (VGCs) result in further efflux of K^+, and concurrently Ca^{2+} starts to diffuse into the cell (Faden et al., 1989).

At this point a number of cellular repair processes will be initiated, including activation of Na^+-K^+ pump to restore the ionic gradient (Giza and Hovda, 2001). This requires significant energy generation, thus a short period of hyperglycolysis ensues, lasting for some minutes to hours (Giza and Hovda, 2001). The increasing Ca^{2+} concentration is sequestered by the mitochondria, but the Ca^{2+} here inhibits oxidative metabolism (Choe et al., 2012). Therefore, energy demands must be met by glycolysis, which in turn results in lactate accumulation and thus decreased ATP production (Giza and Hovda, 2001). Consequently, the cell enters a phase of metabolic suppression and widespread depression. Ca^{2+} is also implicated to be involved in enzyme activation and initiation of apoptotic pathways (Morgan and Curran, 1986). Immediate decreases in Mg^{2+} have also been found following a TBI; this has been shown to slow down recovery while, pre-treatment of animals with magnesium has resulted in improved post-traumatic neurological outcome (McIntosh et al., 1988; Vink et al., 1987). Persistent emotional, cognitive, and somatic symptoms observed following a mTBI likely reflect disruptions in the neurochemical cascades described and their interaction with downstream effectors.

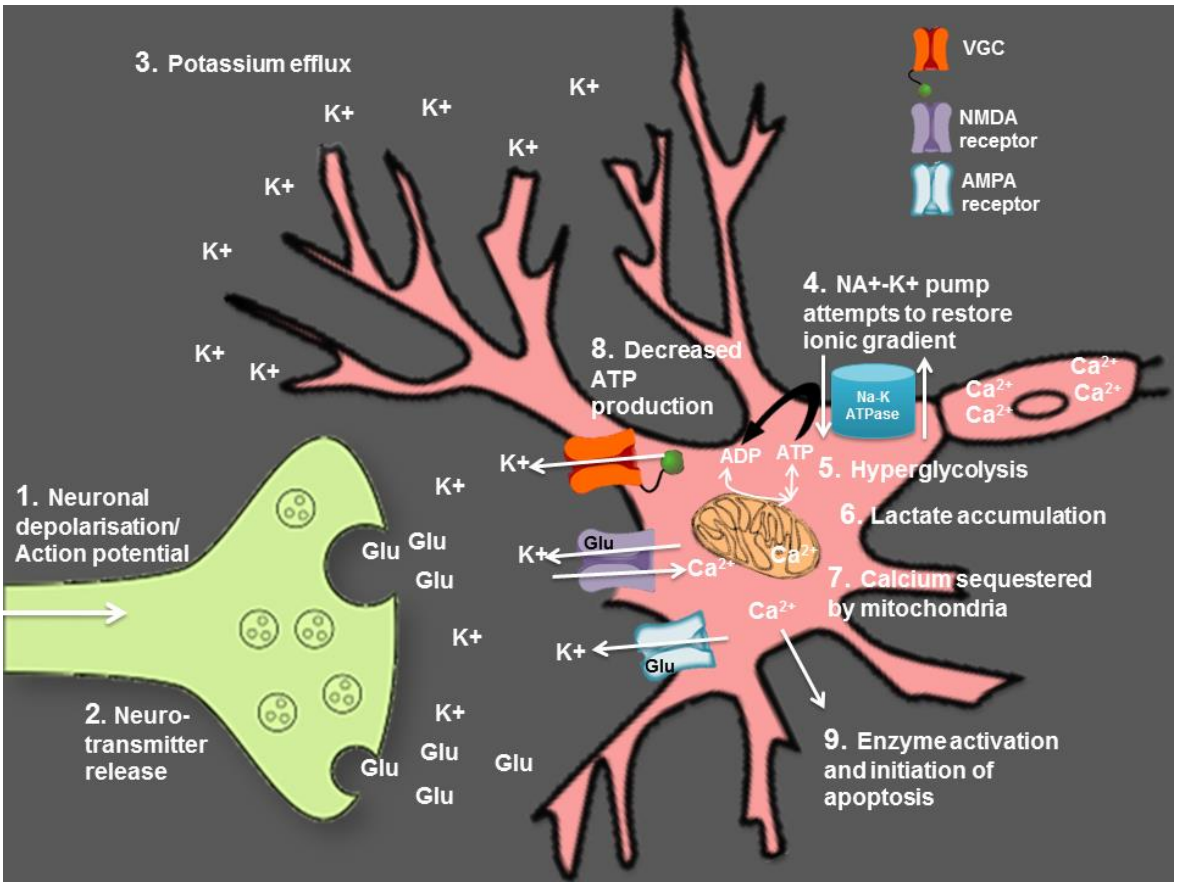

Figure 1.4 The neurochemical cascade initiated following a TBI.

1.1.7 Repeated mTBIs

Given the mild and mostly transient symptoms after mTBI/concussion and the absence of apparent structural changes, affected individuals often return to play or duty following a single mTBI and are at risk of repeated injury (Meehan and Bachur, 2009; Rosenfeld et al., 2013). A study looking at retired professional football players reveals 61% had suffered at least one concussion, and 24% had experienced 3 or more, while a soldier may be exposed to an average of 50 blast exposures during deployment (Guskiewicz et al., 2005; Petrie et al., 2014). Little is known about the underlying mechanisms of injury, or the synergistic effect of repeated exposures, although suggestions have been made linking repeated mTBI with persistent symptomology and cognitive decline (Fehily and Fitzgerald, 2016; Nichols et al., 2015). Repetitive blast exposures have been associated with hypometabolism in the cerebellum of veterans, delayed recovery, and/ or worsened symptomology of post-TBI sequelae (Carr et al., 2016; Hue et al., 2014; Nichols et al., 2016; Ojo et al., 2015).

Concern have been raised about repeated head injuries possibly leading to latent pathological processes complicit in neurodegenerative disorders such as chronic traumatic encephalopathy (CTE) following repeated mTBIs (McKee and Robinson, 2014; Ojo et al., 2015). Currently CTE is only diagnosable upon a neuropathological exam post-mortem, characterised by perivascular accumulation of tau protein in deep cortical sulci (McKee et al.,

2015). Thus far, no definitive link has been made between blast exposure and CTE, but there are some limited case studies of service members with a likely CTE diagnosis, although it is uncertain if their injuries were purely blast-related or they had a history of other mTBIs (McKee and Robinson, 2014). While post-mortem studies of professional boxers and football players have revealed neurofibrillary tangles at sulcal depths and superficial cortical layers, with retrospective descriptions of early behaviour and mood changes, followed by later cognitive decline (McKee et al., 2013). Suggestions that repeated mTBIs may lead to increased vulnerability and decreased threshold for damage is an active area of research both clinically and pre-clinically.

1.1.8 The role of sex-differences in TBI

There is little consensus currently on the topic of TBI and sex-specific differences. Some studies implicate sex is a physiological factor, which can confer advantages or disadvantages to the injury cascade following TBI, while other findings do not support a sex-specific difference. The biggest discrepancy appears to be between clinical and experimental data. In experimental TBI females appear more resilient, for example showing a delayed and lower magnitude of TBI associated neurodegeneration compared to males, including reduced oedema formation and blood brain barrier leakage, reduced inflammatory response and apoptotic cell death (Günther et al., 2015; Kupina et al., 2003; O'Connor et al., 2006). The female hormones oestrogen and/or progesterone are implicated to play a role in the sex-specific differences observed in animal models (Roof and Hall, 2000). The neuroprotective effects of progesterone has been demonstrated in several studies, where it has been shown to positively affect inflammation, oedema and plasticity following a TBI (Maghool et al., 2013; Si et al., 2014; Stein, 2013). While oestrogen has been shown to decrease anxiety-like behaviour (Hiroi et al., 2011).

In clinical studies women appear more vulnerable to TBI both as a result of military-related combat and in contact sports; they reportedly suffer more concussions and report more post-concussive symptomology (Bazarian et al., 2010; Brickell et al., 2017; Colvin et al., 2009; Dick, 2009; Iverson et al., 2011). Some studies indicate that female veterans are more likely to have depression, or PTSD comorbid with depression, while male veterans are more likely to have PTSD with substance use disorder (Iverson et al., 2011; Pugh et al., 2016). While other studies do not find sex-specific differences in reporting of post-concussive symptoms, or believe it has only a limited influence in the military population (Jackson et al., 2016; Rogers et al., 2014). Given the increasing number of females in military combat and participation in contact sports, concerns about women's health following a mTBI have been raised and further research is warranted.

1.2 Monoaminergic and neuropeptidergic neurotransmission

The underlying pathomechanisms of mTBI as a result of contact sports or exposure to explosive devices are poorly understood. Furthermore, the causal mechanisms which lead to persistent neuropsychiatric symptoms in some affected individuals are also relatively unknown. However, some aspects of the neurochemical changes immediately following injury have been delineated, including the indiscriminate release of neurotransmitters, mainly EEAs (as described before, see Figure 1.4). Other studies have also observed changes in other neurotransmitters including increased acetylcholine (ACh) and decreased NA levels in the seconds to hours post-TBI (Robinson et al., 1990; Saija et al., 1988; Tanaka et al., 1997). In fact, decreasing ACh with A-4 (a bis tertiary amine derivative of hemicholinium-3), significantly reduces behavioural deficits following TBI (Robinson et al., 1990), while administration of NDMA-antagonists (blocking the action of EEAs) also limited neurological dysfunction and had beneficial behavioural effects (Faden et al., 1989). Given these findings and the established role of some classical neurotransmitters in emotional and cognitive impairments, including symptoms that are characterised under PCS and PTSD, a role for neurotransmitter systems in the pathobiology of mTBI is likely.

Furthermore, a role for neuropeptides and their receptors have also been considered relevant in relation to mood disorders (Hökfelt et al., 2003; Holmes et al., 2003; Nemeroff and Vale, 2005), and as a possible therapeutic and/or biomarker following TBI (Donkin et al., 2011; Duan et al., 2013; Liu et al., 1994; Lorente et al., 2015).

1.2.1 Noradrenaline and Serotonin

A vast body of literature has firmly associated dysfunction in the classical neurotransmitter systems using noradrenaline (NA) or serotonin (5-hydroxytryptamine, 5-HT) to stress/mood disorders (Mathew et al., 2008; Millan, 2006). Both systems have been implicated in the early hypotheses of depression, and are the targets of well-established pharmacological treatments of mood/anxiety disorders, such as major depression (Millan, 2006) and PTSD (Davis et al., 1997; Krystal and Neumeister, 2009).

Noradrenaline. NA is synthesised in the bilateral locus coeruleus (LC) nuclei, containing the noradrenergic cell group A6 in the brainstem (Dahlstrom and Fuxe, 1964), expressing the rate limiting enzyme tyrosine hydroxylase (TH; Figure 1.5a) (Nagatsu et al., 1964). The LC innervates virtually all regions of the central nervous system (CNS), including the prefrontal cortex, both the dorsal and ventral hippocampal formation, and the hypothalamus (Ungerstedt, 1971). NA has been shown to play a key role in a number of processes including mood regulation, arousal, and as a stress hormone in the fight or flight response (Aston-Jones and Cohen, 2005; Berridge, 2008; Bremner et al., 1996; Goddard et al., 2010; Harro and Oreland, 2001). Upregulated NA levels are implicated in PTSD symptomology, associated with lack of sleep and increased nightmares of the traumatic event (Blanchard et al., 1991).

Serotonin. The raphe nuclei neurone cell groups, B1-B9, are the principal neurones that give rise to spinal and extensive serotonergic forebrain projections (Dahlstrom and Fuxe, 1964; Steinbusch, 1981) and express the rate-limiting enzyme tryptophan hydroxylase 2 (TPH2, Figure 1.5b) for 5-HT synthesis (Walther et al., 2003). 5-HT is involved in a vast array of physiological processes, including regulating sleep, appetite, pain and mood. Chronic stress has been shown to alter the level of 5-HT in the brain, specifically a reduction in the level of 5-HT is found in the plasma and CSF of some depressed patients (Gao et al., 2008). A reduction in 5-HT transporter binding has also been found in post-mortem brains of depressed patients (Maes, M and Meltzer, 1995).

The levels of these monoamines and their metabolites have been examined in various animal models for TBI and models for a number of other stress/mood disorders. Increased levels of NA and DOPAC, concurrent with decreased 5-HT and HVA were found in the hippocampus of stressed animals (exposure to predator scent) in a PTSD model (Wilson et al., 2014). While halothane exposure, restraint and swim stress (consecutively) were reported to elevate NA, 5-HT and dopamine concentrations in the hippocampus after 7 days, but not after 1 h (Harvey et al., 2006). Furthermore, several studies have also reported elevations in the levels of the monoamine biosynthetic enzymes TH (Chang et al., 2000; Tóth et al., 2008; Yan et al., 2001) and TPH2 (Chamas et al., 1999, 2004; Donner et al., 2012; Shishkina et al., 2007), respectively, following exposure to various types of stressors.

Significant increases in TH protein levels in the adrenal medulla and nucleus tractus solitarii of the brainstem, with associated elevated plasma NA levels at 6 h post-exposure to blast, have been reported (Tümer et al., 2013). Changes in monoamine turnover have also been reported in other TBI models, including decreased 5-HT and NA, and increases in the metabolite 5-HIAA ipsilaterally, six days after unilateral ventrolateral, cortical lesions (Finklestein et al., 1983). Pappius and Dadoun (1987) reported decreased 5-HT levels bilaterally after 1 d, and increased 5-HIAA, which remained elevated up to 10 d, following a unilateral, focal cortical freezing or heat lesion. The same group also demonstrated an increase in 5-HT synthesis in the cortex, hippocampus, and DRN using the same model (Tsuiki et al., 1995).

There has been some success in the treatment of PTSD with pharmacological interventions. Treatment with an alpha-1 adrenergic antagonist, Prazosin, has shown efficacy for decreasing nightmares, sleep disturbances and other PTSD related symptoms in veterans (Raskind et al., 2003). Currently two FDA approved pharmacological drugs of PTSD are both selective serotonin reuptake inhibitors (SSRIs) (Davidson et al., 2001; Marshall et al., 2001). Taken together, these studies suggest that the monoamine systems react in response to psychosocial stress and various types of TBI. However, thus far there have been no successful pharmacological interventions to improve post-TBI sequelae, despite attempts with several antidepressants. Lack of success has been explained by confounding factors, such as gender, timings of intervention post-injury, and heterogeneity of TBIs (Osier and Dixon, 2016).

1.2.2 Classical neurotransmitter and neuropeptide synthesis and release

Classical neurotransmitters like the monoamines NA and 5-HT are synthesised in nerve cell bodies and nerve terminals from diet derived amino acids by several enzymatic steps (Figure 1.5). Commonly, they are then stored mainly in small synaptic vesicles, partly accumulated close to the active zone of the synapse, and released upon low frequency stimulations (Iversen et al., 2009). Classical neurotransmitters are recycled; there are reuptake mechanisms upon termination of their action (Iversen et al., 2009).

Neuropeptides are formed in the cell body or dendrite of a neurone (Strand, 1999). Their synthesis takes place on ribosomes, and thereafter they are packaged into large dense core vesicles (LDCV) in the Golgi apparatus. The prepropeptide form is cleaved by specific enzymes to the bioactive form. LDCVs, sometimes also containing a classical neurotransmitter and one or more other neuropeptides, are transported from the cell body to the axonal terminal through fast axonal transport. Their release usually requires high frequency or burst firing for exocytosis, but both neuropeptides and neurotransmitters are Ca^{2+} dependant. Upon release they can bind to receptors on the cell soma, dendrites, axons and nerve endings, but have no reuptake mechanism and are inactivated by extracellular peptidases (Hökfelt et al., 2003; Lundberg, 1996).

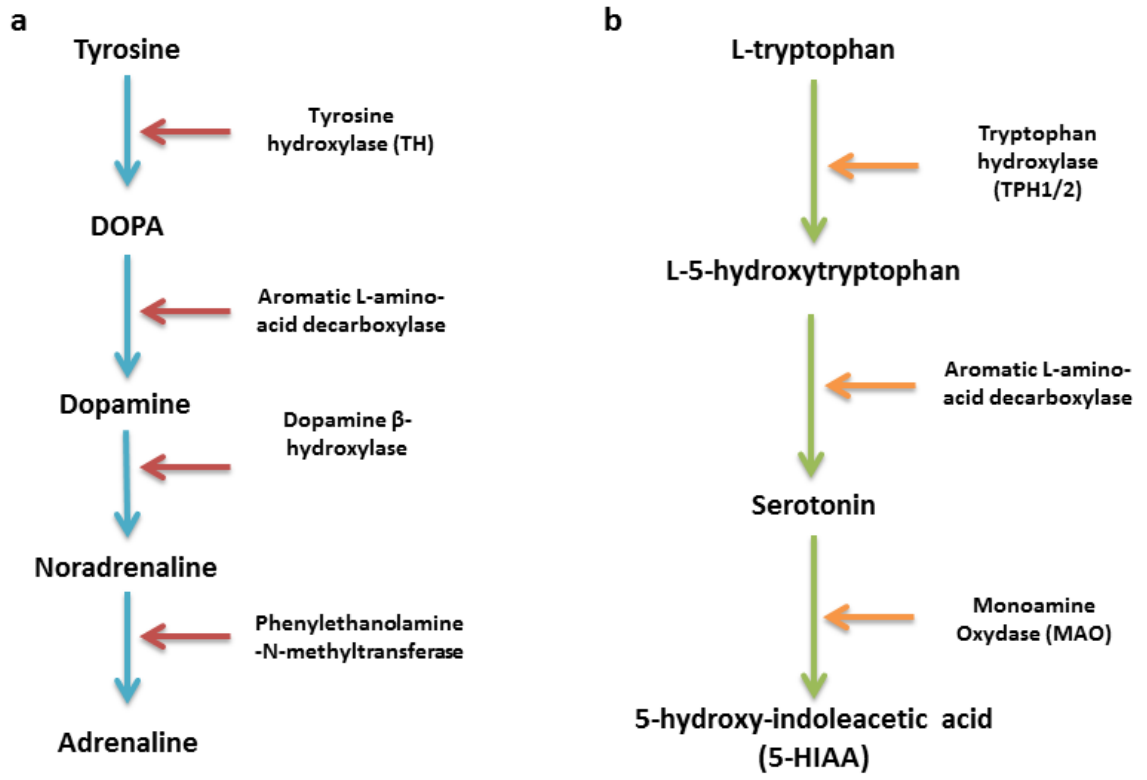

Figure 1.5 Biosynthesis of noradrenaline (a), and serotonin (b).

1.2.3 Galanin and galanin receptors

Galanin is a 29 amino acid (30 in human) neuropeptide (Lang et al., 2015; Tatemoto et al., 1983). It is of considerable interest given its co-localisation with monoamines. In the rat it is co-expressed in around 80% of the noradrenergic neurones in the LC and in 40% of the serotonergic neurones in the DRN (Fuxe et al., 1998; Holets et al., 1988; Melander et al., 1986b). A potential therapeutic role for galanin following TBI has been suggested. Following a focal injury, using a fluid percussion model, rats treated with galanin prior to injury had significantly less motor deficits compared to their vehicle-TBI injured counterparts (Liu et al., 1994). Galanin has also been implicated to play a role in mood/stress disorders (Juhasz et al., 2014; Karlsson and Holmes, 2006; Sciolino and Holmes, 2012; Weiss et al., 2005). It is believed to act as a neuromodulator inhibiting post-synaptic neurotransmitter release, or exert a stimulatory effect on exocytosis (Lang et al., 2015).

Galanin signalling is hypothesised to be 'silent' under basal conditions and to be distinctly increased following stress exposure such as hyperactivity of the LC neurones. In support of this theory, upregulated levels of both TH and galanin have been reported following acute stress in the LC (Holmes et al., 1995; Kuteeva et al., 2010; Sweerts et al., 1999). Furthermore, galanin inhibition of LC neuronal activity at the soma-dendrite level, may prevent generation of an overactive LC system (Pieribone et al., 1998; Vila-Porcile et al., 2009). This may lead to receptor supersensitivity and increased susceptibility to stress, possibly representing an early stage of depression (Harro and Oreland, 2001). Galanin also modulates 5-HT signalling (Fuxe et al., 1998), and inhibits 5-HT and NA release (Yoshitake et al., 2003). Galanin signalling has also been shown to modulate the stress response in an animal model of PTSD (Kozlovsky et al., 2009).

Galanin mediates its actions through three G-protein coupled receptor subtypes, galanin receptor 1–3 (GalR1–3, Figure 1.6). These receptors are widely expressed across the peripheral nervous system (PNS) and CNS, in distinct and partly over-lapping regions (Waters and Krause, 2000). The receptors show high interspecies homology, but low homology to each other (Liu et al., 2010).

GalR1 is believed to inhibit release of neurotransmitters by acting through $G_{i/o}$ type G-proteins, leading to reduced cyclic AMP (cAMP) concentrations, activating inwardly rectifying K^+ channels, and stimulating mitogen activating protein kinase (MAPK) activity (Branchek et al., 2000; Lang et al., 2015). GalR1 mRNA expression as determined by *in situ* hybridisation (ISH) was found in a number of forebrain regions including the hypothalamus, amygdala, ventral hippocampus and thalamus, and also in the brainstem including the LC (O'Donnell et al., 2003). In the hypothalamus GalR1levels are found increased in females compared to males and varies across the oestrus cycle (Faure-Virelizier et al., 1998). Upregulation of GalR1 in the vPAG has also been found in rats exposed to a chronic mild stress (CMS) model, and after knocking down GalR1with siRNA all parameters of the depressive-like phenotype were rescued (Wang et al., 2016).

GalR2 signals through both $G_{i/q}$ and $G_{i/o}$ type G-protein (Branchek et al., 2000; Lang et al., 2015). Activation through the $G_{i/q}$ subunit triggers the phospholipase C (PLC) pathway, mobilises intracellular Ca^{2+}, and has a stimulatory effect on exocytosis (Howard et al., 1997). While acting through the $G_{i/o}$ subunit it has an inhibitory effect on exocytosis. GalR2 is widely distributed in the brain, including in the hypothalamus, hippocampus, amygdala and cerebellar cortex and also in peripheral tissues such as the heart, intestines, ovaries and prostate (O'Donnell et al., 2003; Waters and Krause, 2000). This receptor is thought to mediate 'anti-depressive' effects of galanin, given that it is decreased following CMS (Kuteeva et al., 2008; Lu et al., 2005). Also, stimulation by the GalR2 agonist M1896 in the DRN has been shown to increase 5-HT release in the hippocampus (Mazarati et al., 2005).

Activation of *GalR3*, like GalR1 produces a hyperpolarising response by activating inwardly rectifying K^+ channels consistent with coupling to $G_{i/o}$ type G-protein (Branchek et al., 2000; Lang et al., 2015). GalR3 is also widely distributed in peripheral tissues such as the spleen, liver, heart and kidney, along with several brain regions including hypothalamus, cerebral cortex, caudate putamen, and medulla (Waters and Krause, 2000). Interestingly, GalR3 knockout mouse have been reported to exhibit an anxiety-like phenotype (Brunner et al., 2014).

It should be noted that there are some species-specific differences with regards to galanin and its receptor distribution in the CNS. Particularly with respect to the brainstem, while GalR1 has been observed in many cells within the adjacent median raphe nucleus in the rat, in mice it is not detected (Larm et al., 2003). Furthermore, GalR1 and GalR2 are present in the LC and DRN in the rat, and GalR3 levels are very low, contrasting a distinct GalR3 expression in the human LC (Barde et al., 2016; Le Maître et al., 2013).

NA and 5-HT extensively innervate forebrain regions, play key roles in limbic circuits, and interact with other neurobiological mediators such as galanin. If these systems are dysregulated they likely cause deficits in attention, memory, arousal, sleep regulation, mood, and anxiety, leading to much of the symptomology described in PCS and PTSD in affected populations. This thesis will focus on these systems following a mild blast-induced TBI.

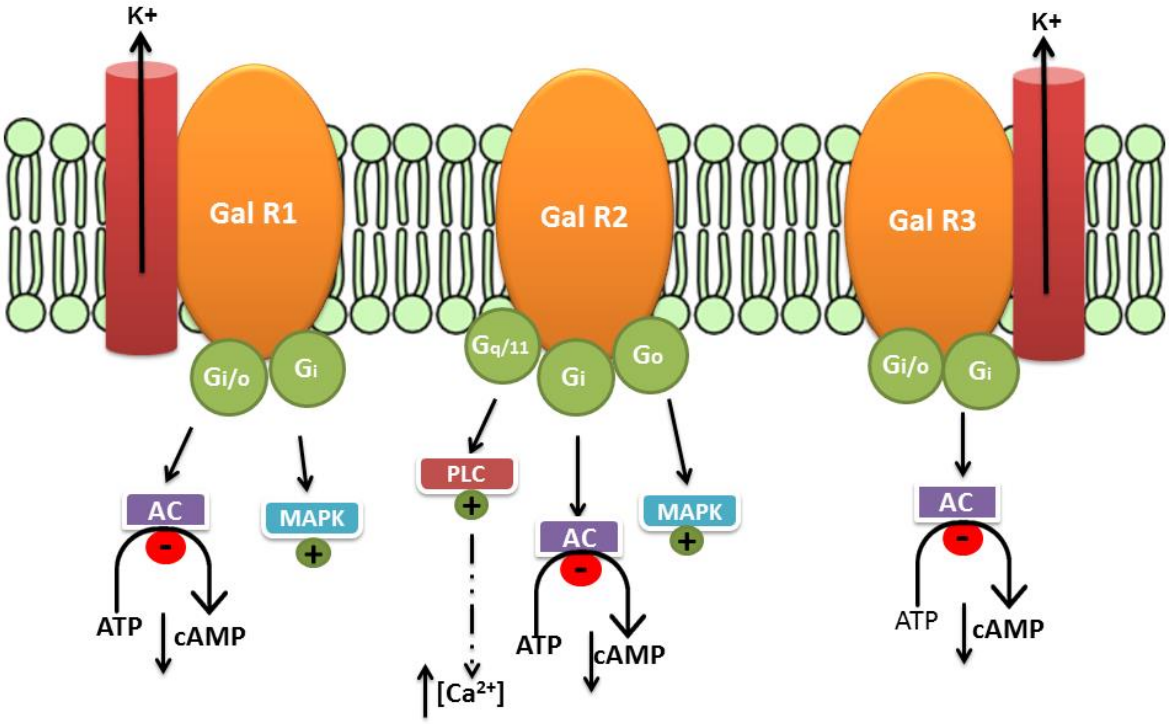

Figure 1.6 Schematic representation of the three galanin receptor subtypes (GalR1-3).

2 AIMS

Aim 1: To study behavioural and neurochemical changes following a single blast exposure, focusing on the monoamine systems ***(Paper I).***

Aim 2: To study neurochemical changes following a single blast exposure, focusing on the galanin system ***(Paper II).***

Aim 3: To investigate the effects of multiple blast exposures on the monoamine and galanin systems in the brainstem ***(Paper III)***

Aim 4: To compare findings across two different rodent models of blast exposure in two different laboratories ***(Paper III)***.

Aim 5: To explore the role of sex-specific differences on the monoamine systems following a single blast exposure ***(Paper IV)***.

3 METHODOLOGY

3.1 Animals

In all the studies included in this thesis were male or female Sprague Dawley rats (Taconic, Ry, Denmark) for experiments at Karolinska Institutet (KI, in Stockholm, Sweden), and (Charles River Laboratories, Wilmington, MA) for experiments carried out at the Uniformed Services University (USU, Bethesda, Maryland, USA). The weight ranged between 250-350 g. All work performed at KI were in accordance with the Swedish National Guidelines for Animal Experiments, and approved by the Stockholm Animal Care and Use Ethics Committee (Stockholm Norra Djurförsöksetiska Nämnd) with the following ethical permit numbers: N422/10, N248/11, and N81/13. The study carried out at USU was performed according to a protocol approved by the Institutional Animal Care and Use Committee at USU with the following ethical permit number: APG-14-911.

The animals were deeply anaesthetised by using a 2.4 ml/kg intra-peritoneal injection consisting of a mixture of 1ml Dormicum® (5 mg/ml Midazolan; Roche), 1 ml Hypnorm® (Janssen) and 2 ml of distilled water (Studies I and II). Anaesthesia methodology was modified for study III and IV: here rats were anaesthetised using a 4% isoflurane-air mixture for 6 min (KI: Janssen, Stockholm, Sweden; USU: Forane, Baxter Healthcare Corporation, Deerfield, IL, USA). For the repeated exposure study animals were anaesthetised for 3 min approximately 30 min later. The animals were then exposed to the blast exposure.

3.2 Exposure models

3.2.1 Primary blast exposure model

A validated 1.5 m long metal blast tube, designed by Clemedson in 1955 and since modified for studies on rodents, was used for the primary blast exposure (Clemedson et al., 1956; Säljö et al., 2000). Swedish army plastic explosive containing explosive m/46, 86% pentaerythritol tetranitrate (PETN) and mineral oil was used with a NONEL ignition (Nobel, Sweden). Anaesthetised animals were placed in a rigid metallic holder that protects all parts of the body except the head. Furthermore, the holder prevented acceleration movements of the head relative to the rest of the body. The rats were mounted into the tube, in a transverse prone position and placed at a distance of 1 m from the charge. Five grams of the explosive was then detonated, triggering a simple blast Friedlander-type wave at the surface of the animal with a peak pressure of 550 kPa and a duration of 0.2 ms. The right side of the rat's head faced the charge. Animals were housed on site, and all experimental manipulations, including the anaesthesia, exposure and sacrifice were carried out in the same laboratory (Study I-IV). This model is referred to as the 'KI model' (Figure 3.1).

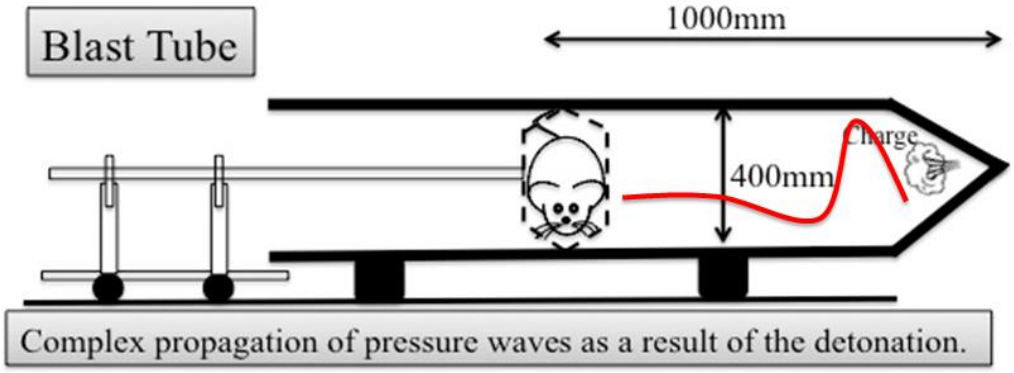

Figure 3.1 A schematic presentation of the blast tube.

3.2.2 Shock tube exposure model

The shock tube is a 525 cm long, 30 cm in diameter, horizontally mounted, circular steel tube, and divided into a 75 cm compression chamber separated from a 450 cm expansion chamber by polyester Mylar sheets (Du Pont Co, Wilmington, DE, USA) (Elsayed, 1997; Long et al., 2009b). The peak pressure obtained varies depending upon the number and thickness of the Mylar sheets placed. The relationship between Mylar sheet thickness and the pressure produced is linear. Anaesthetised rats, wearing body protection, were placed in the shock tube holder in a transverse prone position, with the right side of the animal facing the incident blast overpressure. The average peak pressure was 88.9 kPa, with a 8-9 ms positive phase duration. Here, animals were transported from USU (Bethesda, MD, USA) to Walter Reed Army Institute of Research on the day of the exposures (WRAIR; Silver Spring, MD, USA) and transported back when the exposures were concluded (Study IV). We have referred to this shock tube as the "WRAIR model" (Figure 3.2).

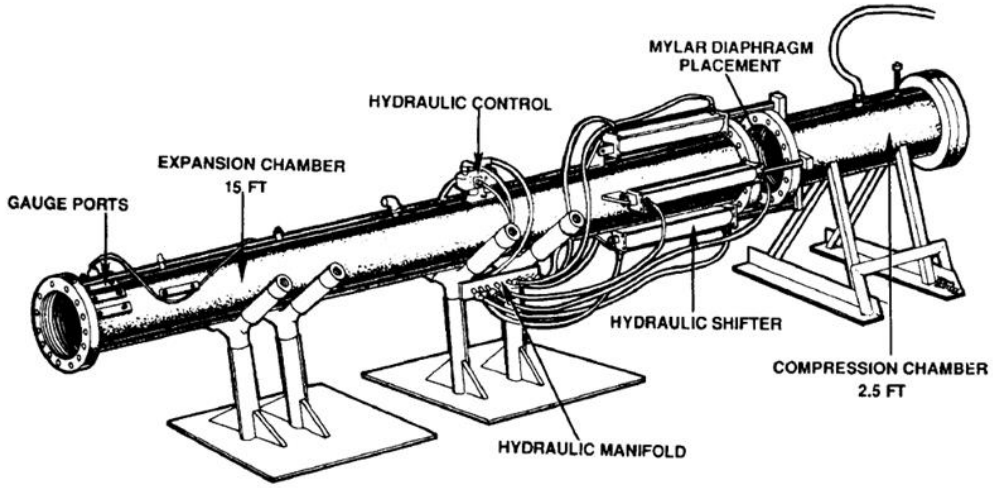

Figure 3.2 A schematic presentation of the shock tube.
Reproduced with permission from (Elsayed, 1997).

3.3 Analysing gene and protein expression

3.3.1 Immunohistochemistry

Flouro Jade B (FJ) stains for degenerating neurones. Sections were air dried, fixed in 4% formaldehyde, and rinsed in phosphate-buffered saline (PBS). The sections were subsequently dipped in dH_2O, followed by 10 min incubation in potassium permanganate (KMnO4), and then rinsed with dH_2O. Slides were then soaked for 30 min in FJ solution (Fluoro Jade B, Merk Millipore AG310, Darmstadt, Germany), washed in dH_2O, and placed on a 50^{o}C hot plate, dipped in xylene and thereafter mounted with Entellan (Merck, Darmstadt, Germany).

White matter damage was evaluated by monitoring β-amyloid precursor protein (APP) accumulation. Sections were fixed in ice cold methanol, shortly dipped in ice cold acetone, then rinsed in PBS. Slides were then incubated in a humid chamber overnight with a solution of 0.3% Triton, 5% bovine serum albumin, and 0.1% sodium azide in 0.01M PBS and a rabbit poly-clonal antibody against APP (#51-2700, dilution 1:400; Life Technologies, Stockholm, Sweden). The following day all sections were rinsed in PBS and incubated for 1 h with 0.01 M PBS, 0.1% sodium azide, 0.3% Triton, and processed with an Alexa Fluor 488 conjugated anti-rabbit IgG (Jackson ImmunoResearch, dilution 1:400; Suffolk, UK). Sections were rinsed and mounted in a mixture of glycerol and PBS (1:3), then cover-slipped. Slides were viewed in a microscope equipped with epifluorescence (Eclipse E600, Nikon, Tokyo, Japan).

Leakages of blood vessels were evaluated using a secondary rat antibody. Sections were processed as described for APP, including overnight incubation in a humid chamber, but not exposed to any primary antibody. The following day sections were rinsed in PBS and incubated with CY2-conjugated donkey anti-rat antibody (#712-225-153, dilution 1:100; Jackson ImmunoResearch, Suffolk, UK). Sections were then rinsed and processed as described for APP staining.

3.3.2 In situ hybridisation (ISH)

The rats were anesthetised and decapitated, the brains removed, placed on dry ice and stored at -70^{o}C until use. Serial coronal, 14 µm thick sections at the level of the LC (bregma -10.52 – - 9.16 mm) and DRN (- 8.30 – - 7.30 mm; coordinates according to Paxinos and Watson (Paxinos and Watson, 2007)) were cut using Cryo-Star HM 560 M (MICROM International GmbH, Heidelberg, Germany) and thaw mounted on super frost slide (Thermo Scientific, Stockholm, Sweden).

Oligonucleotides complementary to rat mRNA for TH, TPH2, galanin, neuropeptide tyrosine (NPY) and aromatic acid decarboxylase (AADC) (Table 1) were labelled with deoxyadenosine 5'triphosphate α–P^{32} (Perkin Elmer, Boston, MA) at the 3′-end using terminal deoxynucleotidyltransferase (Thermo Scientific, Waltham, MA). The labelled

oligoprobes were purified using ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech, Piscataway, NJ). Sections were air-dried and incubated with the oligonucleotide probe for 18 h at 42^{o}C (Dagerlind et al., 1992).

RNA probes specific to GalR1 were prepared from rat hypothalamus cDNA (Table 1). The PCR fragments were then subcloned into PCR1II-TOPO vector (Life Technologies, Carlsbad, CA) and transcribed using T7 and SP6 RNA polymerases to generate sense and antisense RNA probes.

For the Ribo-ISH the sectioned tissues were first fixed for 10 min in cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.4, followed by 5 min in 1 x PBS, DEPC-treated water, and 0.1 M HCl, 3 min in PBS (twice), 20 min in 0.25% acetic anhydride in 0.1 M triethanolamine, 3 min in PBS (twice), and then dehydrated for 2 min in ascending ethanol concentrations and stored at -20°C until pre-hybridisation. Sections were prehybridised using 50% deionized formamide (pH 5), 50 mM Tris-HCl (pH 7.6), 25 mM ethylene-diamine-tetraacetate (EDTA, pH 8), 20 mM NaCl, 0.25 mg/ml yeast tRNA, 2.5 x Denhardt's solution for 4 - 6 h at 55ºC, and subsequently hybridised in a humidified chamber overnight (16 h) at 55ºC.

Labelled probes were diluted to a concentration of 1.0 x 10^{6} c.p.m./200 µl in a solution containing 50% deionized formamide (pH 5), 0.3 M NaCl, 20 mM dithiothreitol, 0.5 mg/ml yeast tRNA, 0.1 mg/ml poly-A-RNA, 10% dextran sulphate and 1 x Denhardt's solution. Following hybridisation, sections were washed with constant stirring as follows: 30 min in 1 x SSC at 55°C (twice), 1 h in 50% formamide/0.5 x SSC at 55°C, 15 min in 1 x SSC at 55°C, 1 h in RNase A buffer at 37°C, 15 min in 1 x SSC at 55°C (twice), dehydrated in ascending alcohol series (2 min each) and finally air dried.

Post-hybridisation, all sections were rinsed in 1 x SSC, 5 x 30 min at 55^{o}C followed by 1 h at room temperature (RT), dehydrated with ascending ethanol concentrations, air-dried and dipped in liquid photo emulsion NTB2 at RT(Kodak, Rochester, NY).

The optimal exposure time was determined by exposing the slides to imaging plates (BAS-SR Fujifilm, Tokyo, Japan) for 24 h at RT, which were scanned using a phosphoimager (Fuji BAS 3000, Tokyo, Japan). Based on this data, TPH2 slides were developed after 72 h, galanin and TH typically after one week, and GalR1 after 8-12 weeks using D19 developer (Kodak) and AL-4 fixative (Kodak) and mounted in glycerolphosphate buffer.

Dark field photomicrographs were captured using a microscope (Nikon Eclipse E-600; Nikon, Tokyo, Japan), connected to a digital camera (Digital Sight, U1; Nikon). The images were analysed according to the mean grey density (MGD) of the messenger ribonucleic acid (mRNA) signal in the regions of interest (ROIs), using ImageJ 1.48 (National Institutes of Health, Bethesda, MD).

Table 3.1. Probes used for ISH and qPCR.

Probe	Gene Bank accession no.	Primers
Primers used for oligo in situ hybridization		
TH	NM_012740	GCG CTG GAT ACG AGA GGC ATA GTT CCT GAG CTT GTC
TPH2	NM_017139	TCC TCC GTC CAA ATG TTG TCA GGT GGA TTC AGC GTC ACA ATG GTG GTC
Galanin	NM_017139	GGTGCACAGTGGGTGTGGTCTCAGGACTGCTCT ATGCCAGGCAGGCTGTCGAGGGCCCCGGCCTCT GTGCGGACGATATTGCTCTCAGGCAGGGGTACA CCCGAGCCCCAGAGTGGCTGACAGGGTTGCAACCAACAGGAGCCAGGC TTGTCAATGGCATGTGGGCCCAGAAGGTAGCCA
NPY	NM_012614	TTGATGTAGTGTCGCAGAGCGGAGTAGTATCTGGCCATGTCC
AADC	NM_012614	TACCAGCTGATATATCGGCTGATAGACCTGCCGTATCTCCC
Primers used to synthesize riboprobes		
Rat GalR1	NM_012958	TTTGTGGACTGGCACCTCCTTCCGA (Fwd) TTTCCTGGGTTCTTTGGAGGCCCA (Rev)
Primers used for qPCR		
Rat Galanin	NM_017139	TGCAACCCTGTCAGCCACTC (Fwd) TGTCGCTAAATGATCTGTGGTTGTC (Rev)
Rat GalR1	NM_012958	AGGCTTACGTGGTGTGCACTTTC (Fwd) GCCATGATATGCCAAATACCACAA (Rev)
Rat GalR2	NM_019172	CATCGTGGCGGTGCTTTT (Fwd) AGCGGGAAGCGACCAAAC (Rev)
Rat GalR3	NM_019173	AGGAAGATGAGGGCAAAG (Fwd) GAAGATGACATGAAACCAGG (Rev)
Rat GAPDH	NM_017008	GGCACAGTCAAGGCTGAGAATG (Fwd) ATGGTGGTGAAGACGCCAGTA (Rev)

3.3.3 Quantitative polymerase chain reaction (qPCR)

The following regions were dissected using anatomical landmarks (Paxinos and Watson, 2007): prefrontal cortex (PFC), occipital cortex (CX), entorhinal cortex (ERC), hypothalamus (Hyp), amygdala (Amg), dorsal and ventral hippocampal formation (dHiFo and vHiFo). Both ipsi (left)- and contra (right)-lateral regions were dissected, except for Hyp (Figure 3.3 a-d). Tissue blocks including LC and vPAG/DRN were removed using tissue punches (AgnTho's AB, Lidingö, Sweden, Figure 3.3 e-f).

Total RNA was extracted using RNeasy plus mini kit (Qiagen, Düsseldorf, Germany) for the dissected regions and using RNAqueous micro kit (Life Technologies) for the tissue punches. After reverse-transcription using iScript select cDNA synthesis kit (Bio-Rad, Berkley, CA), qPCR was performed using the SYBR green PCR master mix and ABI Prism® 7000 sequence detection system (Life Technologies) under the following conditions: 50°C for 2 min, 95°C for 10 mins, followed by 45 consecutive cycles of 95°C for 15 sec and 60°C for 1 min. Relative gene expression was determined by the $2^{-\Delta\Delta}C_T$ method and normalised to the housekeeping gene GAPDH (primer sequences used are shown in Table 1).

3.3.4 Radioimmunoassay

Both ipsi- and contralateral sides of the following regions were rapidly dissected: PFC, Amg, dHiFo, and vHiFo. The LC and vPAG were punched out as described with qPCR (Figure. 3.3). The tissue samples were weighed, extracted in acetic acid, boiled, homogenised, centrifuged, lyophilised and dissolved before analysis, as previously described (Theodorsson and Rugarn, 2000). Rat galanin-like immunoreactivity was analysed using antiserum RatGala4 raised against conjugated synthetic rat galanin, measuring a single immunoreactive component corresponding to rat galanin (1-29) (HPLC), but also included immunoreactive molecules eluting right after the void volume (gel-filtration chromatograms) (Theodorsson and Rugarn, 2000). Competitive RIA of cholecystokinin (CCK) was performed as described for galanin using antiserum Ab 2609 (Rehfeld, 1987).

3.4 **Neurochemical analyses**

3.4.1 High-performance liquid chromatography (HPLC)

Chemicals required for HPLC included: NA hydrochloride, DA hydrochloride, 5-HT hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), potassium chloride, phosphate monobasic monohydrate, sodium phosphate dibasic, sodium acetate, citric acid, octanesulfonic acid sodium salt and methanol, which were obtained from Sigma-Aldrich (St. Louis, MO, USA). EDTA-2Na was purchased from Dojindo (Kumamoto, Japan).

The following regions were dissected using the appropriate landmarks as described before: PFC, Hyp, dHiFo, vHiFo, CX and ERC (Figure 3.3 a-d). Both ipsi (left)- and contra (right)-lateral regions were isolated (except for the Hyp), put into marked plastic vials, and placed on dry ice.

Dissected brain tissue samples (1-10 mg) were mixed at a ratio of 1:10 (w/v) with 0.2 M perchloric acid including 100 µM EDTA-2Na, and homogenised in a glass-pestle micro-homogeniser. Following standing for 3 min on ice, the homogenates were centrifuged for 15 min at 12,000 g at 4°C. The supernatants were aspirated and mixed with 1 M Na-acetate buffer, pH 3 at a ratio 5:1 (v/v) and filtered through a 0.22 µm centrifugal filter for 4 min at 12,000 g at 4°C. The filtrates were stored at -80°C before HPLC analysis.

Concentrations of NA, DA, and 5-HT in the brain tissue samples were determined by HPLC with electrochemical detection as described by (Kehr and Yoshitake, 2006). The HPLC system consisted of a HTEC500 unit (Eicom, Kyoto, Japan), and a CMA/200 Refrigerated Microsampler (CMA Microdialysis, Stockholm, Sweden) equipped with a 20 µl loop and operating at 4°C. The potential of the glassy carbon working electrode was +450 mV versus the Ag/AgCl reference electrode. The separation was achieved on a 200 x 2.0 mm Eicompak CAX column (Eicom). The mobile phase was a mixture of methanol and 0.1 M phosphate buffer (pH 6.0) (30:70, v/v), containing 40 mM potassium chloride and 0.13 mM EDTA-2Na. The chromatograms were recorded and integrated by use of a computerised data

acquisition system Clarity (DataApex, Prague, Czech Republic). The detection limit (signal-to-noise ratio = 3) for NA, DA and 5-HT was 0.05 nM, that is, 0.75 fmol in 15 µl injected onto the column respectively.

Concentrations of DOPAC, HVA, and 5-HIAA were determined by a separate HPLC system with electrochemical detection (HTEC500). The potential of the glassy carbon working electrode was +750 mV versus the Ag/AgCl reference electrode. The separation was achieved on a 150 x 3.0 mm Eicompak SC-5ODS column (Eicom). The mobile phase was a mixture of methanol and 0.1 M citrate/0.1 M sodium acetate buffer solution (pH 3.5) (16:84, v/v) and contained 210 mg/l octanesulphonic acid sodium salt and 5 mg/l EDTA-2Na. The detection limit (signal-to-noise ratio = 3) for DOPAC, HVA and 5-HIAA was 2 nM, that is, 10 fmol in 5 µl injected onto the column. The chromatograms were recorded and integrated by use of the computerised data acquisition system Clarity (DataApex).

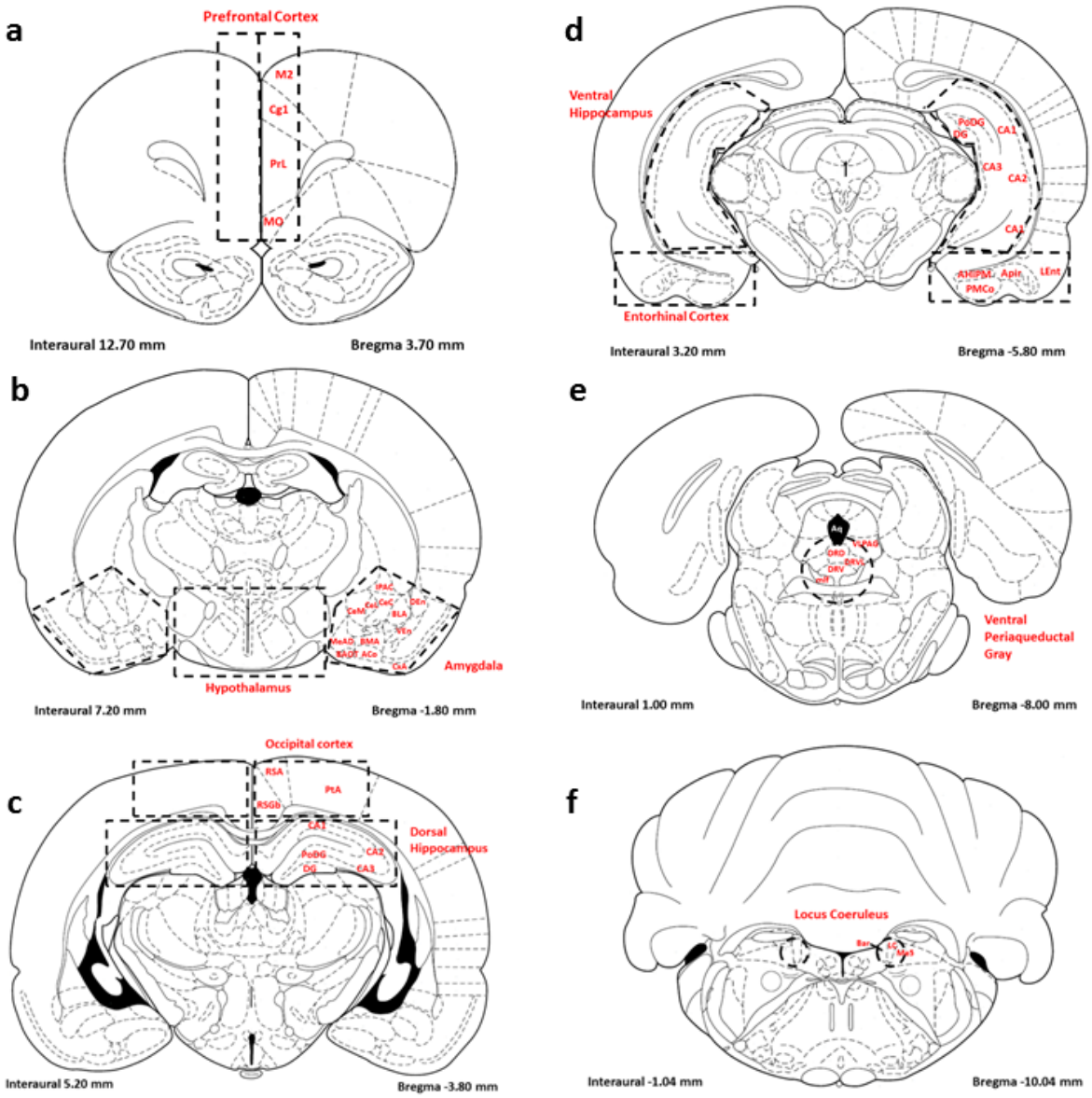

Figure 3.3 Schematic presentation of brain regions dissected for qPCR, RIA, and HPLC analysis (a-f). Images repreinted with permission from The Paxinos and Watson atlas (Paxinos and Watson, 2007).

3.5 Serum analyses

3.5.1 Enzyme-linked immunosorbent assay (ELISA)

All assays were run in accordance to manufacturer's instructions. Progesterone (PROG) and estradiol (E2) levels were measured using Rat PROG ELISA Kit and Rat E2 ELISA Kit (both from CUSABIO, Nordic BioSite, Stockholm, Sweden).

Substance P (SP) levels were measured using Biomatik ELISA Kit (Biomatik Corporation, Ontario, Canada). Each sample was diluted 1:2.

Corticosterone (CORT) levels were measured using abcam's Corticosterone ELISA Kit (BioNordika, Stockholm, Sweden), here samples were diluted to 1:100.

S100 levels were determined using CanAG S100 assay (CanAg Diagnostics AB, Gothenburg, Sweden).

The levels of mature BDNF were analysed with Biosensis RapidTM Mature BDNF ELISA Kit (VWR, Stockholm, Sweden). Samples were diluted 1:100.

3.6 Behavioural tests

3.6.1 Forced Swim Test- FST

Anxiety-/depression-like behaviour was measured using the FST (Porsolt et al., 1978) at 1 d pre-exposure (baseline) and 1 d, 14 d, and 35 d post-exposure. Animals were individually placed in a vertical plastic cylinder (50 cm height, 18 cm diameter) containing water to a height of 30 cm, at a temperature of 25 ± 0.5^{o}C. Animals were exposed to two swimming sessions, twenty-four hours apart: a 10-min pre-test, and a 5-min test. The total duration of immobility and climbing behaviour were recorded during the second test day. Immobility was defined as floating passively in an upright position in water, with only small movements necessary to keep the head above the water surface, typically only one paw movement at a time (Detke et al., 1995). Climbing was defined as vigorous fore-paw movements directed toward the walls of the cylinder (Figure 3.4).

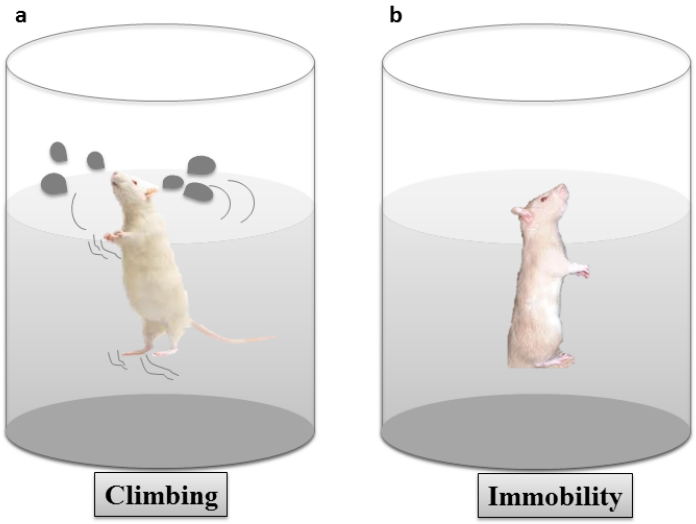

Figure 3.4 The forced swim test. The different behaviours that can be measured are illustrated (a) climbing and (b) immobility behaviour.

3.7 Statistical analysis

GraphPad Prism version 5 or 6 (GraphPad Software, CA) was used to perform all statistical analyses using ANOVA followed up by Tukey-Kramer Multiple Comparison Test and independent t-tests. All data are presented as the mean ± SEM, where statistically significant data is highlighted: * p <0.05, ** p <0.01, *** p <0.001 **** p <0.0001.

4 RESULTS AND DISCUSSION

4.1 Paper I: The monoamine systems and mbTBI

The underlying pathomechanisms of a blast-induced mTBI are still relatively unknown. Exposure to blast appears to cause persistent and lingering neuropsychiatric symptoms including mood disorders and anxiety in a subset of those injured. Monoamine projections from the brainstem and midbrain, particularly noradrenaline in the locus coeruleus and serotonin in the dorsal raphe, play a key role in modulating the functions of forebrain regions. Dysfunctions in these systems have been associated with mood/anxiety disorders implicating these regions and neurotransmitters in the pathomechanism of neurobehavioral symptoms after blast-induced mTBI. In this study we sought to identify behavioural and neurochemical changes in the monoamine systems following a blast-induced mTBI.

We first examined sections from both the brainstem and forebrain regions for degenerating neurones, blood vessel leakage and APP accumulation to ascertain, if our model did indeed produce a mild TBI. Lack of neuropathological findings with this series of staining indicates it is indeed, in line with other studies of mbTBI (Elder et al., 2012; Kamnaksh et al., 2012; Kwon et al., 2011).

We then explored changes in the transcript levels of the monoamine synthesising enzymes in the brainstem, and the levels of the neurotransmitters NA, DA, and 5-HT, along with their metabolites DOPAC, HVA, and 5-HIAA, in a number of forebrain regions. We found an acute and transient increase in TH transcript levels bilaterally in the LC, and of TPH2 in the mid/caudal, but not rostral, DRN as early as 2 h post-exposure. The levels of TH and TPH2 returned to sham levels at 7 and 3 d, respectively. With regard to the neurotransmitter levels and those of the metabolites, the most striking changes were confined to the NA system. We measured significant increases in NA levels 1 d post-exposure in a number of forebrain regions (i.e., projection areas of the LC-NA neurones, Table 1A). DA was also increased, but elevations were limited to the hippocampus. No changes were observed in 5-HT levels, but its metabolite 5-HIAA was decreased in the PFC. All these changes occurred acutely, that is at 1 d post-exposure. At 7 d, two metabolites of the catecholamine system, DOPAC and HVA, were decreased in the cortex and hippocampus (Table 1B).

Table 1. HPLC Raw Data Values for Monoamines (A), and Their Metabolites (B), at 1 D and 7 D Post-Exposure

(A)	*Monoamines [pg/mg]*											
Group	*NA*				*DA*				*5-HT*			
Mean±SEM	*Sham 1 d*	*Exposed 1 d*	*Sham 7 d*	*Exposed 7 d*	*Sham 1 d*	*Exposed 1 d*	*Sham 7 d*	*Exposed 7 d*	*Sham 1 d*	*Exposed 1 d*	*Sham 7 d*	*Exposed 7 d*
Hypothalamus	2176±153.4	2516±282.7	1468±176.7	1236±93.1	241.4±58.0	368.0±31.01	199.5±28.2	208.2±43.4	261.5±47.1	334.9±34.3	263.2±40.2	288.8±31.3
Prefrontal cortex	322.5±12.8	320.3±13.4	291.8±23.5	288.1±30.8	78.1±7.3	87.6±8.4	154.8±47.1	120.0±24.4	237.9±24.9	242.1±11.9	186.5±15.5	180.3±16.7
Occipital cortex	238.3±10.5	**293.3±8.3*** **	196.2±13.2	194.1±18.8	21.8±3.5	25.1±3.7	5.8±0.39	6.1±0.7	159.2±13.5	190.2±8.2	72.7±5.3	84.1±9.2
Entorhinal cortex	348.7±22.8	**510.0±59.4***	325.9±21.3	327.7±28.4	193.5±38.7	335.5±82.1	54.7±9.9	46.1±9.1	519.7±46.5	526.6±59.6	183.6±22.0	204.8±22.9
Dorsal hippocampus	295.7±15.5	**399.2±25.0***	232.2±21.9	234.3±19.8	17.1±1.3	**26.4±3.9***	5.9±0.8	8.2±1.0	251.5±20.4	291.4±20.6	112.4±11.5	142.9±10.7
Ventral hippocampus	473.4±17.1	533.9±38.5	266.5±15.9	307.5±15.3	17.7±1.5	**26.5±3.0***	7.0±0.8	7.4±0.6	390.5±31.5	435.2±39.3	125.5±9.6	130.2±9.7

(B)	*Metabolites [pg/mg]*											
Group	*DOPAC*				*HVA*				5-HIAA			
Mean±SEM	*Sham 1 d*	*Exposed 1 d*	*Sham 7 d*	*Exposed 7 d*	*Sham 1 d*	*Exposed 1 d*	*Sham 7 d*	*Exposed 7 d*	*Sham 1 d*	*Exposed 1 d*	*Sham 7 d*	*Exposed 7 d*
Hypothalamus	115.7±11.1	88.9±9.3	88.7±5.8	83.3±9.7	45.7±3.9	36.1±6.9	54.3±7.9	49.5±6.4	760.2±31.9	665.3±42.2	427.7±21.5	418.7±26.4
Prefrontal cortex	43.7±3.5	35.8±2.6	151.0±37.1	125.3±10.2	94.9±9.7	87.8±12.3	114.0±10.7	144.9±21.7	305.1±10.9	**257.9±8.0** **	336.6±26.0	371.5±25.3
Occipital cortex	5.2±0.2	5.7±0.7	8.2±0.5	**6.0±0.5** **	6.3±0.3	7.2±0.9	7.5±0.7	7.2±0.6	172.3±5.2	179.0±6.3	198.3±8.4	184.9±6.5
Entorhinal cortex	49.9±10.7	47.0±14.0	31.1±4.3	22.1±4.3	40.9±8.9	44.0±11.2	33.2±2.3	**18.1±2.5*** **	319.0±23.4	344.8±38.9	327.7±13.4	318.9±24.7
Dorsal hippocampus	6.3±0.5	8.8±1.5	4.0±0.8	3.7±0.4	9.5±0.9	9.0±1.0	9.9±1.1	**5.9±0.5** **	388.2±15.7	384.6±11.4	324.2±25.8	359.4±15.6
Ventral hippocampus	7.0±1.1	4.9±0.3	4.1±0.4	3.8±0.5	6.3±0.4	6.2±0.5	10.4±1.4	11.4±2.0	382.2±16.9	376.6±16.8	336.7±19.0	329.3±13.9

Data are presented as mean±S.E.M.
*$p<0.05$; ** $p<0.01$; ***$p<0.001$.
HPLC, high-performance liquid chromatography; NA, noradrenaline; DA, dopamine; 5-HT, 5-hydroxytryptamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindoleacetic acid; SEM, standard error of the mean.

The increase in monoamine levels suggests a significant release of neurotransmitters, which is followed by compensatory re-synthesis. This would also account for the elevation seen in transcript levels of the monoamine synthesising enzymes and the decrease in metabolite levels.

Many neurones release chemical messengers following stress exposure, including excitatory amino acids such as glutamate, as well as acetylcholine, NA, DA, and 5-HT, in some cases more than one. Monoamine turnover has previously been examined in a number of studies looking at TBI, PTSD, and other trauma and stress-related models, and the studies report selective and specific changes in these systems (Faden et al., 1989; Pappius and Dadoun, 1987; Tanaka et al., 1997; Tsuiki et al., 1995; Wilson et al., 2014).

Taken together our findings are in line with the literature, where the LC-NA system appears to be distinctly sensitive to stress (Bremner et al., 1996; George et al., 2013; Korf et al., 1973; Usher, 1999). Furthermore, increased TH and TPH2 transcript levels in response to various stressors have also been extensively reported on (Chamas et al., 1999, 2004; Rusnák et al., 1998; Yan et al., 2001).

We also evaluated functional changes in anxiety-/depression-like behaviour with the FST, during a 5-min test session ran at 1 d pre-exposure (baseline) and 1 d, 14 d, and 35 d post-exposure (Figure 4.1). As expected, no behavioural differences were found pre-exposure across the groups: control, sham, and exposed. However, 1d post-exposure the exposed group exhibited decreased immobility relative to the control and sham groups (Figure 4.1a). The values for the climbing behaviour were also highest in the exposed group, but this was not statistically significant (Figure 4.1b). Similar climbing and immobility behaviours were observed across the groups for remaining test sessions.

FST has been used to assess learned helplessness following a TBI in several studies with mixed findings, some have reported no changes (Jones et al., 2008), and others have seen increased immobility (Milman et al., 2005). Here, we have interpreted the reduced immobility behaviour immediately following exposure as hyperarousal, based on previous observations that the most anxious animals as evaluated by the elevated plus maze, would spend the least time immobile in the FST (Estanislau et al., 2011). Furthermore, treatment with antidepressants that increase the availability of NA reduces immobility behaviour by increasing time spent climbing (Detke et al., 1995; Detke and Lucki, 1996). Hence it is likely that the widespread elevation in NA levels we observed in the forebrain regions is responsible for the significant reduction in immobility behaviour.

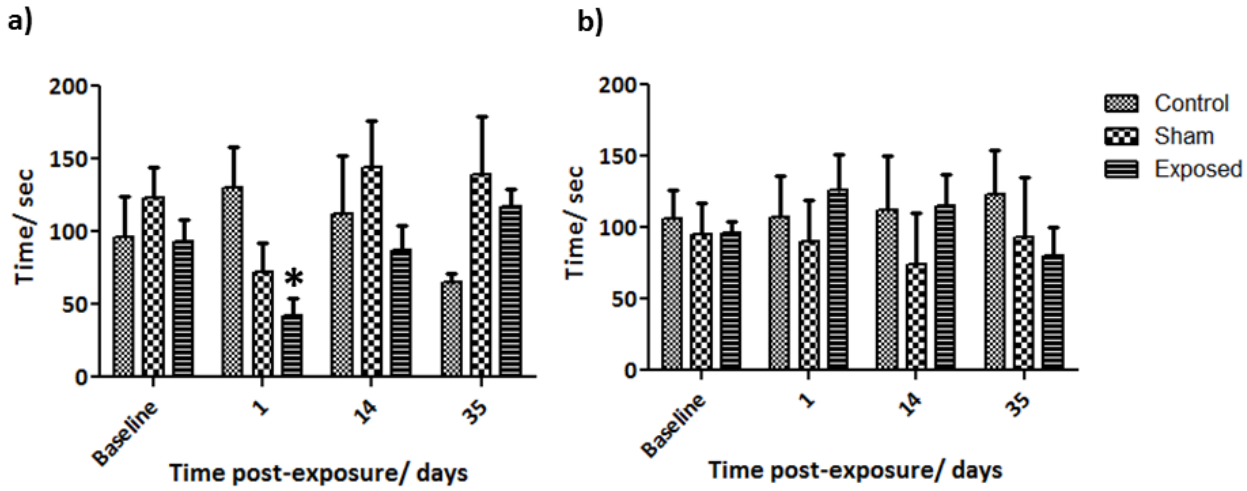

Figure 4.1 Time of immobility (a) and climbing (B) behaviour in the forced swim test measured at 1 d pre-exposure (baseline), and 1 d, 14 d, and 35 d post-exposure.
* $p < 0.05$.

4.2 Paper II: The galanin system and mbTBI

Co-existence of more than one neurotransmitter in neurones has now been well established (Agoston and Conlon, 1986; Hökfelt et al., 2003, 1986; Nemeroff and Vale, 2005). The neuropeptide galanin is co-localised with both NA and 5-HT in the brainstem and midbrain, and similarly innervates the forebrain regions (Fuxe et al., 1990; Melander et al., 1986a; Xu and Hökfelt, 1997). Galanin has been implicated to play a role in mood/stress disorders, and is believed to act as a neuromodulator modulating neurotransmitter release and/or by acting on postsynaptic sites by signalling through its receptors, galanin GalR1-3 (Branchek et al., 2000; Habert-Ortoli et al., 1994; Lang et al., 2015). In this study we examined changes in galanin and its receptors following a single blast exposure.

The levels of transcripts for galanin and those of its three receptors, also of the galanin peptide itself, were perturbed in the brainstem and forebrain regions following a single blast exposure. A significant upregulation of galanin transcripts were observed as early as 2 h and 1 d post-exposure in the LC and DRN, respectively, and remained elevated until D7, the furthest time-point changes were evaluated. Hence changes in the galanin system in the LC and DRN appeared to be more persistent than TH and TPH2, respectively (Figure 4.2). While in the forebrain, a robust and significant increase in galanin mRNA level was observed at D1 in the dHiFo and vHiFo based on the qPCR analysis. We attempted to visualise this with riboprobe *in situ* hybridisation and autoradiography, but unfortunately even after eight weeks of exposure of the hybridised slides we could not detect a signal.

Accumulating evidence suggests neuropeptides are of particular importance when the nervous system is challenged e.g. by stress or traumatic events, serving to modulate the activity of the co-expressed neurotransmitters (Hökfelt, 1991; Lundberg and Hökfelt, 1986). Hence, the differential regulation of galanin and the monoamine synthesising enzymes

observed in this study are in line with previous observations. Galanin signalling is hypothesised to be 'silent' under basal conditions and primarily increased, when the LC neurones are hyperactive. Consistent with this theory, galanin applied directly on to the LC neurones caused hyperpolarisation, presumably by increased K^+ conductance mediated by activation of GalR1/3 receptors (together with noradrenergic inhibition via alpha2A receptors) at the soma-dendrite level (Pieribone et al., 1998; Seutin et al., 1989; Sevcik et al., 1993; Vila-Porcile et al., 2009). Furthermore, microdialysis sampling of the ventral hippocampus following galanin infusion revealed a long lasting and dose dependant reduction in 5-HT levels in the rat (Kehr et al., 2002).

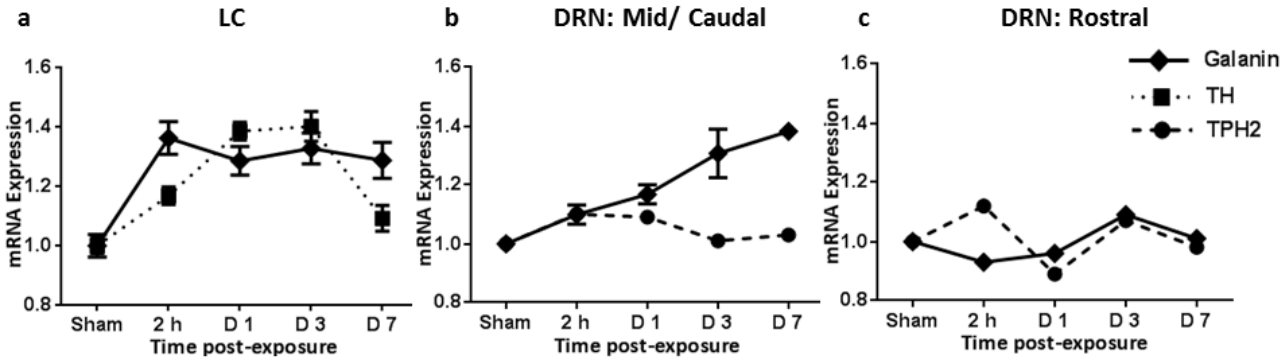

Figure 4.2 Correlation of changes in transcripts for galanin (Paper II)) and for TH and TPH2 (Paper I) in the LC (a) and DRN/vPAG (b, c). Both in the LC and mid/caudal DRN galanin mRNA levels remain elevated at D7.

The wide distribution of galanin receptors (O'Donnell et al., 2003), mediating both inhibitory and stimulatory effects (Lang et al., 2015) contributes to the complex changes in the activity of the neurones expressing these receptors and likely explains some inconsistent findings regarding galanin function in mood/ anxiety disorders. Analysis of the receptor transcripts with ISH in this study revealed several significant changes, in particular a persistent upregulation in GalR1 levels in the mid/caudal vPAG which coincided with a significant decrease in GalR2 in the same region at 7 d post-exposure. In the forebrain, changes were predominately confined to GalR3, which in general decreased acutely, but this decrease was maintained in the vHiFo even after 7 d post-exposure. Also GalR2 was significantly increased in the vHiFo and Amg at 1 d post-exposure.

These differential changes in the receptors in the different brain regions are intriguing given the differential functions of the receptors. GalR1/3 primarily mediates inhibitory actions in the brain, while GalR2 can have both stimulatory and inhibitory effects. In fact stimulation of GalR2 in the DRN by the agonist 1896 has been shown to increase 5-HT release in the hippocampus (Mazarati et al., 2005). Moreover, elevated GalR1 levels in the vPAG, have also been observed in rats exposed to chronic mild stress, which lead to the

onset of depressive-like behaviour (Wang et al., 2016) . This phenotype could be rescued by knocking down expression of this receptor. This is further underscored by recent findings showing increased GalR1 levels (measured by qPCR) in the DRN/vPAG in human depressed suicides versus controls (Barde et al., 2016). These results lend further weight to the present findings observed in the mbTBI. Thus the persistent increased and decreased transcripts of GalR1 and 2, respectively, in the DRN/vPAG suggest that this brain region may be key area for mood control.

The galanin peptide levels in the punched sample containing LC were increased at 3 d, returning to sham levels on 7 d post-exposure. A likely interpretation of this increase is that initially synthesis is increased to compensate for release of the peptide, and that the elevation is a result of accumulation of newly synthesised peptide that will be transported. At this time-point, galanin peptide levels were significantly decreased in the vPAG sample. In the forebrain, galanin peptide levels were significantly increased in the dHiFo, and Amg at 3 d. By 7 d significant galanin decreases versus sham levels were measured in the PFC, vHiFo, and Amg.

We also looked at the transcript levels of neuropeptide Y in the LC, and CCK peptide levels in the brainstem and forebrain regions. Changes were limited to only a significant increase in CCK peptide levels in the LC sample both at 3 and 7 d after blast, suggesting despite the general similarities in these peptide systems, they are differentially regulated and presumably have different functions. The CCK in the LC area is known to be confined to nerve endings (Sutin and Jacobowitz, 1988). Whether the increased levels represent increased or decreased release remains to be explored, but it was early shown that CCK is an excitatory peptide (Dodd and Kelly, 1981).

4.3 Paper III: The monoamine and galanin system following repeated mbTBI

Due to the subtle nature or lack of clinical symptoms following mTBI and the absence of readily available, objective diagnostic tools, individuals who have suffered mTBI typically return to duty or play, where they are at risk of repetitive injuries (Meehan and Bachur, 2009; Petrie et al., 2014). Little is known about the adverse effects of repetitive mTBIs, but it has been suggested that there is a period of increased cerebral vulnerability (ICV) following the injury. ICV is currently interpreted that the brain may not have completely recovered from the initial injury and further insults would have cumulative effects (Vagnozzi et al., 2008; Weil et al., 2014). Hence this study was prompted based on two motivations, first to examine the effects of multiple exposures on the monoamine and galanin systems. Second, to explore how these findings would vary across two commonly used models of blast-induced mTBI.

One day following exposure, sections from the forebrain were evaluated for degenerating neurones using FJ-B histology, and for axonal damage by staining for APP accumulation. No positive staining, hence no evidence of cell degeneration, or signs of axonal injury were detected in rats following either of the single or repeated exposure in either model. This is consistent with previous findings that, while exposure to mild blast results in functional changes as evident in behavioural deficits in both models, there do not appear to be structural changes (Ahmed et al., 2013; Kawa et al., 2014; Kwon et al., 2011). This could be due to the difference in outcome measures because for example Budde and colleagues have found gross changes in the hippocampus when using diffusion tensor imaging (DTI), which they related to significant deficits in memory function in the Morris Water Maze (Budde et al., 2013). Moreover, Kamnaksh *et al.*, (2014) found acute subcortical changes after mbTBI also when using DTI.

Post-concussive symptoms following mbTBI and the possibility that symptoms may be compounded by repeated exposures have been extensively reported on in the literature (Kennedy et al., 2010; Marion et al., 2011; Mendez et al., 2013; Carr et al., 2016). Based on our previous findings in Paper I and II, we wanted to examine how the NA, 5-HT, and galanin systems were affected by repeated exposures. Importantly we wanted to examine if these changes could also be replicated in other blast-models, as this could greatly influence the translatability of these findings.

We observed increased transcript levels of TH and TPH2 in the LC and DRN, respectively, and galanin in both regions, consistent with our previous findings in the KI model following a single exposure (Figure 4.3 and 4.4). But we did not find cumulative effects of repeated exposures in either region in the transcript levels of these molecules, in either of the two rodent models used. Furthermore, changes in TH and galanin in the LC were only statistically significant in the KI model, not in the WRAIR model, here only a trend towards an increase was observed (Figure 1). While in the mid/caudal, but not rostral DRN, an increase in the transcript levels of TPH2 and galanin were also observed at 1 d post-exposure. Again, this increase was statistically significant in the KI model for both the single and double exposed groups. While in the WRAIR model, a trend was seen towards an increase in the single exposed vs sham group, but this only became statistically significant after the second exposure to blast over pressure (Figure 4.4).

These observations lend more support to the NA, 5-HT, and galanin systems playing a likely role in the pathobiology of mbTBI and associated persistent neuropsychiatric complaints. The partially consistent findings across the two models are promising, suggesting these changes are not unique to the KI model but likely a feature of blast exposure. With cautious optimism, this has significant translatability implications given that in the TBI field we struggle with being able to replicate findings across models and eventually translate the findings to the clinic.

The discrepancies in the findings may be explained by the characteristics of the blast models. In the KI model the blast wave has a high peak pressure and short duration, and also the rodents here are exposed to some of the smoke and heat generated by the detonation. While in the WRAIR model the blast over pressure has lower peak but a longer duration, and furthermore animals are transported to and from the facility, thus there is an additional stress component along with the blast.

Our findings are consistent with numerous studies that have demonstrated changes in the NA, 5-HT, and galanin systems in response to various stressors (Chamas et al., 1999, 2004; George et al., 2013; Sweerts et al., 1999; Tóth et al., 2008; Weiss et al., 2007). The lack of cumulative effects following repeated exposure observed in this study could indicate these systems become habituated to the repeated stressor, as shown in previous studies, where TH transcript levels were significantly lower after prolonged immobilisation stress, despite initially increasing, indicating a possible adaptation of the system (Rusnák et al., 1998). Similar observations were made with preprogalanin mRNA, which was initially decreased in the LC, but returned to control levels as restraint sessions increased (Sweerts et al., 1999).

Moreover, perhaps these findings should not be taken to mean that there are no cumulative effects across the system as we have only looked at transcript levels here, but how faithfully these are translated to proteins and to functional changes remain to be evaluated. In fact, Kvetnansky and Sabban (1998) found an increase in TH transcript levels within hours in their stress model, but a marked increase in adrenal TH activity was only evident after seven days. We have only analysed changes after 1 d, perhaps extending to a longer period of time would reveal more.

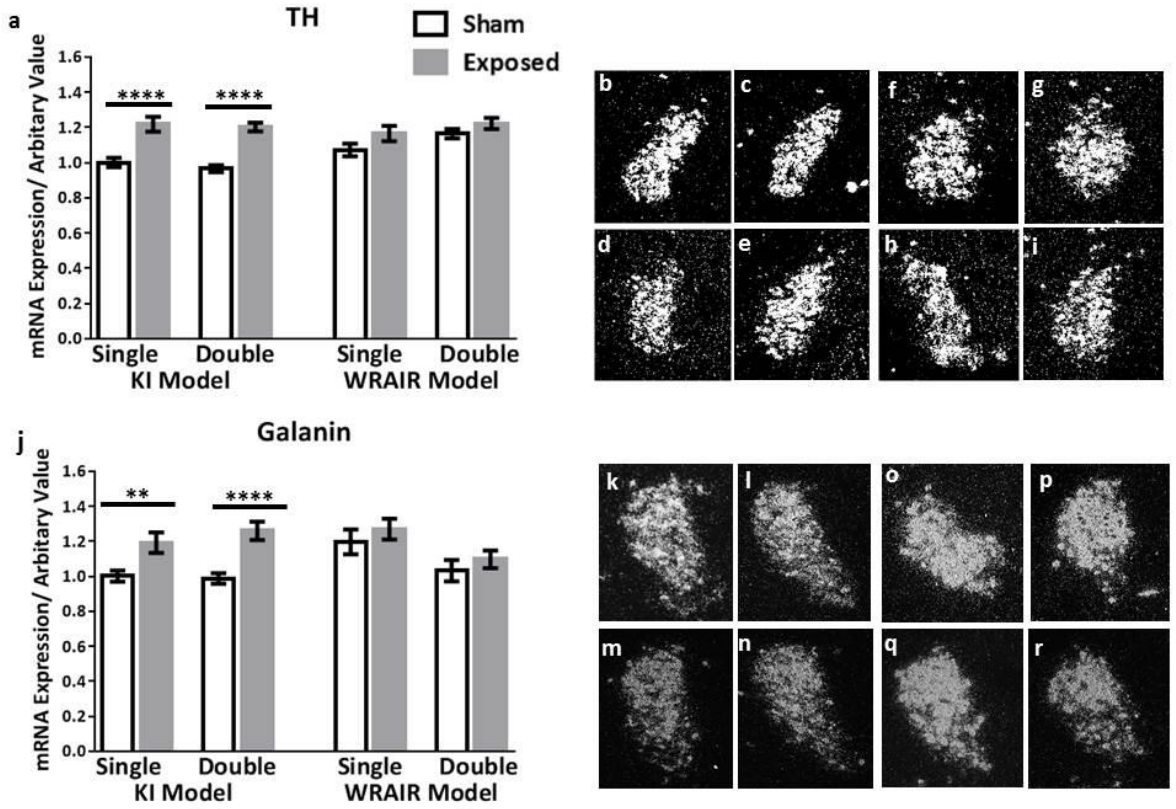

Figure 4.3 ISH analysis of transcript levels of TH and galanin in the LC following exposure to single or double mbTBI, using two different models of TBI, in two different laboratories. (a,j) Quantification of transcript levels of TH (a) and galanin (j) revealed that both were significantly increased bilaterally at 1 day post-exposure in the single and double exposed groups, relative to their respective shams using the KI model. While the same trend was seen in exposed groups vs shams in the WRAIR model, the elevations were not statistically significant in any of the transcripts. There did not appear to be a cumulative effect of repeated exposure in either model.

(b-i, k-r) Representative dark field ISH photomicrographs of emulsion-dipped sections show the distribution and levels of TH (b-i) and galanin (k-r) transcripts levels. TH mRNA levels in the single (b), and double (c) exposed groups in the LC, relative to sham single (d), and double (e) groups using the KI model. (f-i) show TH mRNA distribution and levels in the single (f), and double (g) exposed groups using the WRAIR mbTBI model, and their respective sham groups; single (h), and double (i).Photomicrographs (k-r) show galanin transcript levels: the single (k) and double (l) exposed groups using the KI model, and their respective shams, single (m) and double (n); the single (o), and double exposed (p), and single (q), and double (r) sham groups, using the WRAIR model.

Data are presented as mean ± SEM. (* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001).

ISH, in situ hybridisation; LC, locus coeruleus; TH, tyrosine hydroxylase.

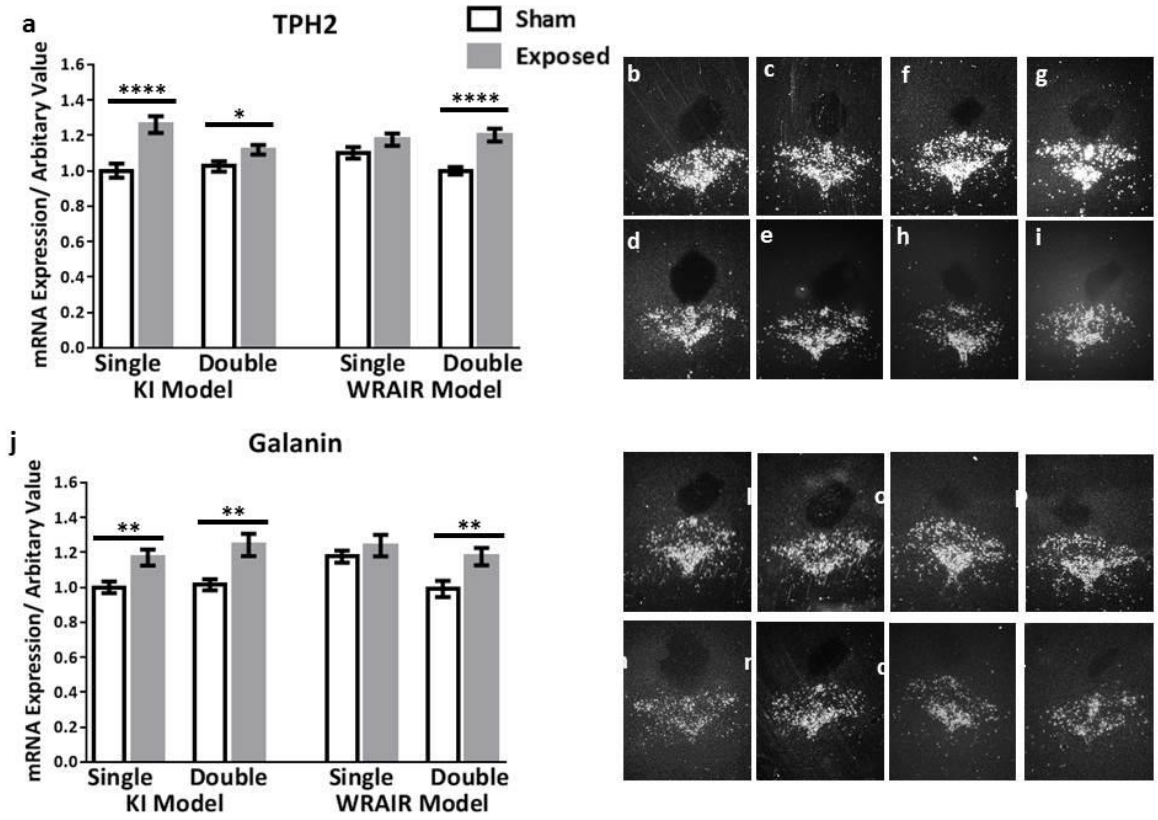

Figure 4.4 ISH analysis of transcript levels of TPH2 and galanin in the mid/caudal DRN following exposure to single or double mbTBI, using two different models of TBI, in two different laboratories. **(a,j)** Quantification of transcript levels of TPH2 (a) and galanin (j) mRNA levels showed a significant increase at 1 day post-exposure in the single and double exposed groups relative to their respective shams using the KI model. While the same trend of an elevation was also observed in exposed groups vs shams using the WRAIR model, this was only statistically significant in the double exposure group. There did not appear to be a cumulative effect of repeated exposure in either model.
(b-i, k-r) Representative dark field ISH photomicrographs of emulsion-dipped sections show distribution and levels of TPH2 (b-i) and galanin (k-r) transcripts. TPH2 mRNA levels in the single (b), and double (c) exposed groups, relative to sham single (d), and double (e) groups using the KI model. (f-i) show single (f), and double (g) exposed groups using the WRAIR model, and their respective single (h), and double (i) sham groups. Photomicrographs (k-r) show galanin transcript levels: the single (k) and double (l) exposed groups using the KI model, and their respective shams, single (m) and double (n); the single (o), and double exposed (p), and single (q), and double (r) sham groups, using the WRAIR model.
Data are presented as mean ± SEM. (* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001).

ISH; in situ hybridisation, DRN; dorsal raphe nucleus, TPH2; tryptophan hydroxylase 2.

4.4 Paper IV: Sex-specific differences in the monoamine systems following a blast-induced mTBI

The number and role of females in the military have recently drastically expanded, along with their participation in contact sports, thus putting females at increased risk of suffering a mTBI (Colvin et al., 2009; Street et al., 2009). Concerns have been raised about possible gender-specific differences after some studies have shown that females suffer more concussions and report more post-concussive symptoms at three months following a mTBI (Bazarian et al., 2010; Colvin et al., 2009; Dick, 2009). Given our findings in Paper I, we set out in this study primarily to explore changes in the monoamine systems following a single blast exposure in female rats, but we also examined changes in select serum biomarkers.

Our examination of forebrain sections for white matter damage, blood vessel leakage and degenerating neurones revealed no differences between exposed and sham female groups. This is consistent with our findings in the exposed males (Kawa et al., 2014), and also with the majority of the blast-induced mTBI literature findings in rodents (Ahlers et al., 2012; Kwon et al., 2011; Long et al., 2009a). We also found no significant changes in body weight between sham and exposed groups of female rats weighed daily a week before and following blast exposure.

Our analysis of TH transcript levels revealed an acute and significant increase in female rats bilaterally in the LC, which normalised by 7d post-exposure (Figure 4.5a), similar to our previous observations in the males shown in Paper I. The transcript levels of the key biosynthetic enzyme TPH2 were also significantly increased at 1d post-exposure, in both the mid/caudal and rostral DRN, and remained elevated even at D7. While the acute findings in the mid/caudal part of the DRN in the females and males are alike, changes in TPH2 transcript levels appeared to be more pronounced and persistent in the females (Figure 4.5b, c).

Our observations are in line with the literature, specifically that the LC-NA system is distinctly activated following stress exposure (Aston-Jones and Cohen, 2005; Korf et al., 1973). Furthermore, previous studies looking at TH levels after chronic repeated restraint stress have found similar elevations in both males and females (Tóth et al., 2008).

However, our findings in the DRN indicate sex-specific differences, with the exposed female group likely more sensitive to the blast exposure. The DRN is a more complex nucleus than the LC given that it has a number of distinct sub-regions (Deakin and Graeff, 1991), which are modulated by a number of different molecules (Fu et al., 2010; Valentino and Commons, 2005). It has been suggested that the sex hormones are also likely to influence post-TBI sequelae (Roof and Hall, 2000). Specifically there are oestrogen receptors in the DRN, and it has also been shown that 5-HT receptors are oestrogen sensitive (Donner and Handa, 2009). Previous studies using rodents have revealed that estradiol administration can have anxiolytic effects in female rats (Hiroi et al., 2011). The

neuroprotective properties of progesterone have been well established, and its therapeutic potential in TBI has been explored (Si et al., 2014). Unfortunately, we were not able to delineate the exact contribution of either hormone in this study given the large spread in estradiol and progesterone hormone levels across the groups. Thus, the rats were likely at different stages of the oestrus cycle.

These findings are particularly relevant in light of clinical reports, where a number of studies have specifically identified that female veterans are more likely to suffer from depression or PTSD comorbid with depression, while males have higher PTSD rates with comorbid substance abuse (Haskell et al., 2011; Iverson et al., 2011; Pugh et al., 2016). Our modest findings would implicate the 5-HT systems in this discrepancy between male and females. Further support to this idea is leant by previous findings in pre-clinical studies suggesting a sex-specific difference in 5-HT metabolism. It is suggested that that females have higher levels of 5-HT synthesising enzymes, higher brain and CSF levels of tryptophan, 5-HT and 5-HIAA compared to males (Carlsson et al., 1985). Furthermore, plasma analysis of tryptophan in major depressed females was found to be lower than males, and correlated negatively with self-rated depression only in the females (Maes et al., 1990).

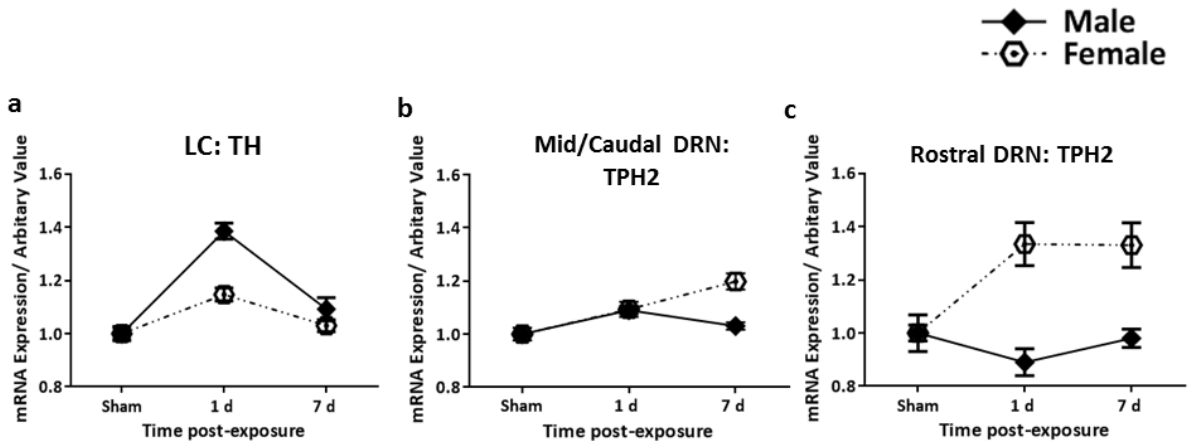

Figure 4.5. **Comparison between female and male rats.** Changes in transcripts for TH and TPH2 in females (present study) and for TH and TPH2 in males (Kawa et al., 2014) in the LC (a) and DRN (b, c) are shown.

Our examination of a number of markers in the serum of female and male rats at 1 post-exposure also revealed some interesting sex-specific differences (Figure 4.6). The levels of CORT and substance P (SP) were both elevated, however only in the exposed females relative to sham animals (Figure 4.6a, c). While a decrease in BDNF levels was only seen in the exposed male group (Figure 4.6b). S100B levels were statistically significantly lower in the female rats, compared to males, regardless of exposure, and also when comparing the exposed and sham female groups (Figure 4.6d).

In the clinical situation an increase in cortisol levels in response to stress is well established (Bierhaus et al., 2003; Lucassen et al., 2014). Maes and colleagues have also shown that oral administration of 5-hydroxytryptophan (5-HTP, the 5-HT precursor), stimulates a high cortisol response, but only in major depressed females, not males (Maes et al., 1987). A likely interpretation of this increase in cortisol may be due to the sensitivity of the hypothalamic-pituitary-adrenal axis to 5-HTP; this is interesting as it directly links changes in the CNS to the periphery. In the TBI field researchers are eagerly looking for biomarkers to objectively diagnose and follow the progression of this disease. Thus, it is of great interest to see how faithfully changes in markers in the periphery could reflect changes in the CNS.

High SP levels in the serum of patients with severe TBI have reportedly correlated with increased mortality (Lorente et al., 2015). Elevated SP serum levels have also been reported in patients with major depression, levels that decline in responders treated with antidepressant (Bondy et al., 2003). SP antagonists have also been used preclinically to alleviate TBI pathology, and it has done so both in male and female rats (Corrigan et al., 2012; Donkin et al., 2011).

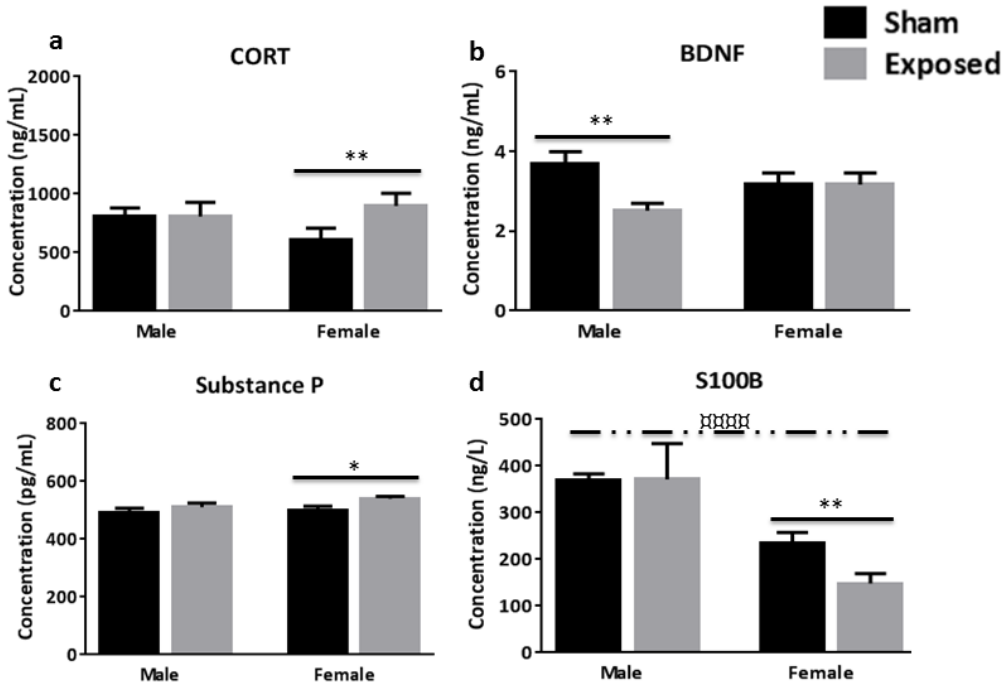

Figure 4.6. **Serum analysis of several markers in female vs males at 1 d following blast exposure.** (a) CORT levels were increased in the female exposed group. (b) BDNF was decrease in the exposed males. (c) Substance P was increased in the female exposed group only. (d) Changes in S100B levels included a decreased in female relative to male groups regardless of exposure.
Data are presented as mean ± SEM. (Independent t-test; * p <0.05, ** p <0.01, ANOVA; ¤ p <0.05, ¤¤¤¤ p <0.0001).

CORT; Corticosterone, BDNF; brain-derived neurotrophic factor, S100B; calcium-binding protein β, ELISA; enzyme-linked immunosorbent assay.

5 GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The injury mechanism of TBI contains the primary injury process caused by the physical forces and occurs immediately after the insult as structural and/or neurochemical changes. The secondary injury process represents the biological response to the injury aimed to restrict and repair the damage. This process can last for an extended period of time (up to weeks and months) and includes metabolic changes, inflammation, etc. Although much has been learnt about metabolic changes and inflammation resulting from TBI, little or no attention has been payed to the potential involvement of neurotransmitters in mediating the injury induced neurobehavioral changes. Initially they can act upon receptors in the central and peripheral nervous system to bring about protective and adaptive changes following the injury. However, when the altered signalling persists, it can be damaging and have pathological consequences.

Taken together the findings of the four studies presented in this thesis implicate the NA, 5-HT and galanin system in the pathobiology of blast-induced mTBI. There is a translational aspect of this data given the partially different response to injury in males and females. In addition, comparing the same outcome measures using two different models of single and repeated mbTBI helps to refine the experimental modelling of the injury and to identify the window of cerebral vulnerability.

Changes in the DRN/vPAG warrant further research, in light of the persistent elevations in TPH2 transcript levels in the females, and of the increased and decreased GalR1 and 2 transcript levels, respectively, in the same region in the males. It would be very interesting to identify, if changes in the galanin receptor levels are similarly affected in females in this region. In fact some preliminary findings in our laboratory indicate sex-specific differences in galanin levels in the brainstem (preliminary observations not included in this thesis). Also sex-specific differences have been described in depressed suicides, specifically GalR1 was found only elevated in males, while GalR3 was elevated in both genders, but more robustly in females (Barde et al., 2016), indicating a translational potential of our findings. Such distinct sex-specific differences may be crucial in developing more efficacious therapy targeted to the specific genders.

Also given the extensive association between galanin and the monoamine systems, combination therapies targeting both systems may have a better therapeutic potential. Although here one needs to cautiously translate these findings from rodents to humans given that galanin receptors subtype distribution appears to be species specific (Le Maître et al., 2013). In the rat GalR1/2 are the dominate receptors in the brainstem, while in humans GalR3 appears to be important (Le Maître et al., 2013). Although, both receptors signal through an inhibitory G-protein subunit, and hence are likely to have similar effects (Lang et al., 2015).

The NA, 5-HT, and galanin extensively innervate forebrain regions and play key roles in limbic circuits. If these systems are dysregulated, as the studies in this thesis suggest, then they may likely cause deficits in concentration, attention, memory, arousal, sleep regulation, mood, and anxiety, leading to much of the symptomology described in PCS and PTSD in affected populations.

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